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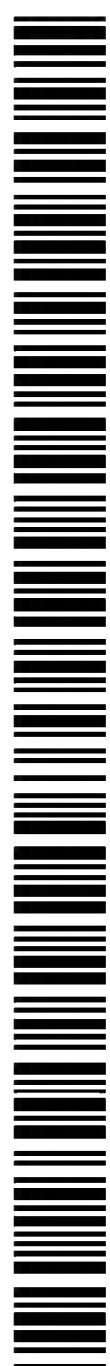
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(54) Title: G1TR BINDING MOLECULES AND USES THEREFOR

(57) Abstract: The present invention provides binding molecules that specifically bind to G1TR, e.g., human G1TR (hG1TR), on T cells and dendritic cells. Binding molecules of the invention are characterized by binding to hG1TR with high affinity, in the presence of a stimulating agent, e.g., CD3, are agonistic, and abrogate the suppression of Teff cells by Treg cells. Various aspects of the invention relate to binding molecules, and pharmaceutical compositions thereof, as well as nucleic acids, recombinant expression vectors and host cells for making such binding molecules. Methods of using a binding molecule of the invention to detect human G1TR or to modulate human G1TR activity, either in vitro or in vivo, are also encompassed by the invention.



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## GITR BINDING MOLECULES AND USES THEREFOR

### *Related Applications*

This application claims the benefit of priority to U.S. Provisional Application, 5 60/665322, filed on March 25, 2005, titled "GITR BINDING MOLECULES AND USES THEREFOR" and U.S. Provisional Application, 60/687265, filed on June 3, 2005, titled "GITR BINDING MOLECULES AND USES THEREFOR", the entire contents of each are hereby incorporated by reference.

### 10 *Background of the Invention*

Members of the tumor necrosis factor and TNF receptor (TNFR) superfamily regulate diverse biologic functions, including cell proliferation, differentiation, and survival. Using differential display to identify T cell mRNAs induced by the synthetic glucocorticoid hormone dexamethasone, Nocentini *et al.* 15 ((1997) *Proc. Natl. Acad. Sci., USA* 94:6216-6221997) identified a mouse cDNA encoding a novel member of the TNFR family. The corresponding gene was designated GITR for glucocorticoid-induced TNFR family-related gene (also known as TNFRSF18). Like other TNFRs, the predicted GITR protein contains cysteine-rich repeats in the extracellular domain. In addition, the intracellular domain of GITR shares 20 significant homology with those of the mouse and human TNFRs, 4-1BB and CD27. Nocentini *et al.* ((1997) *Proc. Natl. Acad. Sci., USA* 94:6216-6221997) demonstrated that the GITR gene is induced in T cells by dexamethasone as well as by other cell-activating stimuli. GITR expression protects T cells from apoptosis induced by treatment with anti-CD3 antibodies, but not by other apoptotic agents.

25 Shimizu *et al.* ((2002) *Nat Immunol* 3:135-42) found that GITR was predominantly expressed on CD4+CD25+ regulatory T cells. However, GITR is also expressed on conventional CD4+ and CD8+ T cells, and its expression is enhanced rapidly after activation. *In vitro* studies have showed that GITR plays a key role in the peripheral tolerance that is mediated by these cells and abrogates the suppressive 30 function of CD4+CD25+ regulatory T cells (Shimizu *et al.* (2002) *Nat Immunol* 3:135-42; McHugh *et al.* (2002) *Immunity* 16:311-23).

The development of agents useful in modulating signaling *via* GITR would be of great benefit.

### 35 *Summary of the Invention*

The present invention provides binding molecules that specifically bind to GITR, *e.g.*, human GITR (hGITR), on cells, such as T cells and dendritic cells. The binding molecules of the invention are characterized by binding to hGITR with high

affinity, are agonistic in the presence of a stimulating agent, *e.g.*, CD3, and abrogate the suppression of T effector (Teff) cells by T regulatory (Treg) cells.

One aspect of the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:1, optionally comprising a leader sequence.

5 In another aspect, the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:66, optionally comprising a leader sequence.

In another aspect, the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:2, optionally comprising a leader sequence.

10 Another aspect of the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:58, optionally comprising a leader sequence.

One aspect of the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:59, optionally comprising a leader sequence.

In another aspect, the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:60, optionally comprising a leader sequence.

15 In one aspect, the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:61, optionally comprising a leader sequence.

In another aspect, the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:62, optionally comprising a leader sequence.

20 One aspect of the invention features a binding molecule comprising the amino acid sequence of SEQ ID NO.:63, optionally comprising a leader sequence.

Yet another aspect of the invention features a binding molecule comprising at least one complementarity determining region (CDR) amino acid sequence selected from the group consisting of: SEQ ID NO.:3, SEQ ID NO.:4, or SEQ ID NO.:19, and SEQ ID NO.:5. In one embodiment, the binding molecule comprises at least two complementarity determining region (CDR) amino acid sequence selected from the group consisting of: SEQ ID NO.:3, SEQ ID NO.:4, or SEQ ID NO.:19, and SEQ ID NO.:5. In another embodiment, the binding molecule comprises at least three complementarity determining region (CDR) amino acid sequence selected from the group consisting of: SEQ ID NO.:3, SEQ ID NO.:4, or SEQ ID NO.:19, and SEQ ID NO.:5.

30 Another aspect of the invention features a binding molecule comprising at least one complementarity determining region (CDR) amino acid sequence selected from the group consisting of: SEQ ID NO.:6, SEQ ID NO.:7, and SEQ ID NO.:8. In one embodiment, the binding molecule comprises at least two complementarity determining region (CDR) amino acid sequence selected from the group consisting of: SEQ ID NO.:6, SEQ ID NO.:7, and SEQ ID NO.:8. In another embodiment, the binding molecule comprises at least three complementarity determining region (CDR) amino

acid sequence selected from the group consisting of: SEQ ID NO.:6, SEQ ID NO.:7, and SEQ ID NO.:8.

Another aspect of the invention features a binding molecule comprising the CDRs shown in SEQ ID NOs.: 3, 4, 5, 6, 7, and 8. In another aspect of the invention  
5 features a binding molecule comprising the CDRs shown in SEQ ID NOs.: 3, 19, 5, 6, 7, and 8.

One aspect of the invention features a binding molecule comprising a heavy chain variable region comprising the amino acid sequence of SEQ ID NO.:1 and further comprising a light chain variable region comprising the amino acid sequence of  
10 SEQ ID NO.:2. Another aspect of the invention features a binding molecule comprising a heavy chain variable region comprising the amino acid sequence of SEQ ID NO.:66 and further comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO.:2. In more than one embodiment, the binding molecule comprises human or substantially human heavy and light chain framework regions. In another  
15 embodiment, one or more human framework amino acid residues are mutated to the corresponding murine amino acid residue. In another embodiment, the constant region comprises an IgG2b heavy chain constant region. In another embodiment, the constant region comprises a human, e.g., human IgG1, heavy chain constant region. . In another embodiment, the binding molecule is altered to reduce effector function and/or  
20 glycosylation. In one embodiment, the binding molecule binds to human GITR. In one embodiment, the binding molecule does not induce apoptosis. In another embodiment, the binding molecule does not block the primary mixed lymphocyte reaction. In yet another embodiment, the binding molecule abrogates the suppression of T effector cells by T regulatory cells. In one embodiment, the binding molecule modulates effector T  
25 cell proliferation. In one embodiment, the binding molecule is murine. In another embodiment, the binding molecule comprises a murine IgG2b heavy chain. In one embodiment, the binding molecule is a humanized antibody. In a further embodiment, the binding molecule is a chimeric antibody. In yet another embodiment, the binding molecule modulates the activity of human GITR. In another embodiment, the binding  
30 molecule attenuates degradation of I- $\kappa$ B in T cells.

Another aspect of the invention features a binding molecule that binds to GITR on human T cells and human dendritic cells and has a binding constant ( $K_d$ ) of  $1 \times 10^{-9}$  or less. In one embodiment, the binding molecule abrogates the suppression of T effector cells by T regulatory cells. In another embodiment, the binding molecule is a  
35 humanized antibody.

Yet another aspect of the invention features a composition comprising a binding molecule of the invention and a pharmaceutically acceptable carrier. In one embodiment, the composition further comprises at least one additional therapeutic agent



for treating cancer in a subject. In one embodiment, the composition further comprises at least one additional therapeutic agent for treating a viral infection in a subject. In another embodiment, the composition further comprises at least one tumor antigen for treating cancer in a subject. In yet another embodiment, the composition further  
5 comprises at least one antigen from a pathogenic agent.

One aspect of the invention features a method for abrogating the suppression of T effector cells by T regulatory cells, comprising contacting human immune cells with a binding molecule of the invention such that the suppression of T effector cells by T regulatory cells is abrogated.

10 Another aspect of the invention features a method for modulating T cell receptor induced signaling in an effector T cell, comprising contacting a cell with a binding molecule of the invention, such that T cell induced receptor signaling in an effector T cell is modulated. In one embodiment, the method modulates the degradation of I- $\kappa$ B. In one embodiment, the T cell is a Th1 cell. In another embodiment, the T cell  
15 is a CD4+ cell. In yet another embodiment, the T cell is a CD8+ cell.

Yet another aspect of the invention features a method for enhancing an immune response in a subject, comprising contacting a cell with a binding molecule of the invention such that that an immune response in a subject is enhanced.

Another aspect of the invention features a method for treating cancer in a  
20 subject, comprising contacting a cell with a binding molecule of the invention such that cancer is treated in a subject. In one embodiment, the type of cancer is selected from the group consisting of: pancreatic cancer, melanomas, breast cancer, lung cancer, bronchial cancer, colorectal cancer, prostate cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system  
25 cancer, esophageal cancer, cervical cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, and cancer of hematological tissues.

Another aspect of the invention features a method for treating an  
30 infection caused by a pathogenic agent in a subject, comprising contacting a cell with the binding molecule of claim 1, such that the infection caused by a pathogenic agent is treated in a subject. In one embodiment, the pathogenic agent is a virus, *e.g.*, selected from the group consisting of: hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV I), herpes simplex type II  
35 (HSV II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, hantavirus, coxsackie virus, mumps virus, measles virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV I), and human immunodeficiency virus type II

(HIV II), any picornaviridae, enteroviruses, caliciviridae, any of the Norwalk group of viruses, togaviruses, such as alphaviruses, flaviviruses, coronaviruses, rabies virus, Marburg viruses, ebola viruses, parainfluenza virus, orthomyxoviruses, bunyaviruses, arenaviruses, reoviruses, rotaviruses, orbiviruses, human T cell leukemia virus type I, human T cell leukemia virus type II, simian immunodeficiency virus, lentiviruses, polyomaviruses, parvoviruses, Epstein Barr virus, human herpesvirus 6, cercopithecine herpes virus 1 (B virus), and poxviruses. In one embodiment, the method is used to treat a chronic viral infection.

In another embodiment, the pathogenic agent is a bacterium, *e.g.*, selected from the group consisting of: *Neisseria spp*, *Streptococcus spp*, *S. mutans*, *Haemophilus spp.*, *Moraxella spp*, *Bordetella spp*, *Mycobacterium spp*, *Legionella spp*, *Escherichia spp*, *Vibrio spp*, *Yersinia spp*, *Campylobacter spp*, *Salmonella spp*, *Listeria spp.*, *Helicobacter spp*, *Pseudomonas spp*, *Staphylococcus spp.*, *Enterococcus spp*, *Clostridium spp.*, *Bacillus spp*, *Corynebacterium spp.*, *Borrelia spp.*, *Ehrlichia spp*, *Rickettsia spp*, *Chlamydia spp.*, *Leptospira spp.*, *Treponema spp*.

Another aspect of the invention features a method for modulating GITR function comprising contacting human GITR with a binding molecule of the invention in the presence of an immunostimulatory agent such that GITR function is modulated.

One aspect of the invention features a binding molecule comprising at least one CDR amino acid sequence selected from the group consisting of: SEQ ID NO.:3, SEQ ID NO.:4, SEQ ID NO.:19, SEQ ID NO.:5, SEQ ID NO.:6, SEQ ID NO.:7, and SEQ ID NO.:8. In one embodiment, the composition further comprises at least one additional therapeutic agent for treating cancer in a subject. In another embodiment, the binding molecule comprises at least one CDR derived from the 6C8 binding molecule. In another embodiment, the binding molecule comprises at least two CDRs derived from the 6C8 binding molecule. In another embodiment, the binding molecule comprises at least three CDRs derived from the 6C8 binding molecule. In another embodiment, the binding molecule comprises at least four CDRs derived from the 6C8 binding molecule. In another embodiment, the binding molecule comprises at least five CDRs derived from the 6C8 binding molecule. In another embodiment, the binding molecule comprises at least six CDRs derived from the 6C8 binding molecule.

Another aspect of the invention features a binding molecule comprising the six CDRs shown in SEQ ID NOs.: 3, 4 or 19, 5, 6, 7, and 8.

Yet another aspect of the invention features a binding molecule comprising a heavy chain variable region comprising the amino acid sequence of SEQ ID NO.:1 and further comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO.:2. In one embodiment, a binding molecule comprises human or substantially human heavy and light chain framework regions. In another

embodiment a binding molecule of the invention comprises human framework regions in which one or more human framework amino acid residues are backmutated to the corresponding murine amino acid residue or are mutated to another amino acid residue. In another embodiment, a binding molecule of the invention comprises a constant region  
5 of an immunoglobulin molecule, *e.g.*, an IgG2b heavy chain constant region. In yet another embodiment, the binding molecule binds to human GITR (hGITR). In one embodiment, the binding molecule does not induce apoptosis. In another embodiment, the binding molecule does not block the primary mixed lymphocyte reaction. In yet another embodiment, the binding molecule abrogates the suppression of T effector cells  
10 by T regulatory cells. In one embodiment, the binding molecule enhances effector T cell proliferation. In another embodiment, the binding molecule neutralizes the activity of human GITR. In yet another embodiment, the binding molecule attenuates degradation of I- $\kappa$ B in T cells.

In one aspect, the invention features a binding molecule that binds to  
15 GITR on human T cells and human dendritic cells and has a binding constant ( $K_d$ ) of  $1 \times 10^{-9}$  or less. In one embodiment, the binding molecule abrogates the suppression of T regulatory cells. In another embodiment, the binding molecule is murine or comprises murine CDRs. In a further embodiment, the binding molecule comprises an IgG2b heavy chain. In one embodiment, the binding molecule is a humanized antibody. In a  
20 further embodiment, the binding molecule is a chimeric antibody.

Another aspect of the invention features, a composition comprising a binding molecule of the invention and a pharmaceutically acceptable carrier. In one embodiment, the composition further comprises at least one additional therapeutic agent for treating cancer in a subject.

25 One aspect of the invention features a method for abrogating the suppression of T effector cells by T regulatory cells, comprising contacting human immune cells with a binding molecule of the invention such that the suppression of T regulatory cells is abrogated.

Another aspect of the invention features a method for modulating T cell  
30 receptor induced signaling in an effector T cell, comprising contacting a cell with a binding molecule of the invention, such that T cell induced receptor signaling in an effector T cell is modulated. In one embodiment, the method modulates the degradation of I- $\kappa$ B. In one embodiment, the T cell is a Th1 cell.

Yet another aspect of the invention features a method for enhancing an  
35 immune response in a subject, comprising contacting a cell with a binding molecule of the invention such that that an immune response in a subject is enhanced.

Another aspect of the invention features a method for treating cancer in a subject, comprising contacting a cell with a binding molecule of the invention such that



cancer is treated in a subject. In one embodiment, the type of cancer is selected from the group consisting of: pancreatic cancer, melanomas, breast cancer, lung cancer, bronchial cancer, colorectal cancer, prostate cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system  
5 cancer, esophageal cancer, cervical cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, and cancer of hematological tissues.

Another aspect of the invention features a method for inhibiting GITR  
10 function comprising contacting human GITR with a binding molecule of the invention in the presence of a stimulating agent such that GITR function is inhibited.

One aspect of the invention features an isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region comprising the nucleotide sequence of SEQ ID NO.:9, optionally comprising a leader sequence.  
15 Another aspect of the invention features an isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region comprising the nucleotide sequence of SEQ ID NO.:67, optionally comprising a leader sequence.

Another aspect of the invention features an isolated nucleic acid molecule comprising a nucleotide sequence encoding a light chain variable region comprising the  
20 nucleotide sequence of SEQ ID NO.:10, optionally comprising a leader sequence.

Yet another aspect of the invention features an isolated nucleic acid molecule comprising a nucleotide sequence encoding at least one CDR selected from the group consisting of: SEQ ID NO.:11, SEQ ID NO.:12 or SEQ ID NO.:65, and SEQ ID NO.:13. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide  
25 sequence encoding at least two CDRs derived from the 6C8 binding molecule. In another embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding at least three CDRs derived from the 6C8 binding molecule.

Another aspect of the invention features an isolated nucleic acid molecule comprising a nucleotide sequence encoding at least one CDR selected from the group  
30 consisting of: SEQ ID NO.:14 SEQ ID NO.:15 and SEQ ID NO.:16. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding at least at least two CDRs derived from the 6C8 binding molecule. In another embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding at least three CDRs derived from the 6C8 binding molecule.

35 One aspect of the invention features an isolated nucleic acid molecule comprising the nucleotide sequences shown in SEQ ID NOs.: 11-16 and SEQ ID NO.:65.

One aspect of the invention features a recombinant expression vector comprising the nucleic acid molecules of the invention. In one embodiment, a



recombinant expression vector comprising a nucleic acid molecule having a nucleotide sequence encoding the binding molecule of the invention is featured. In another embodiment, the invention features a host cell into which the recombinant expression vector of the invention has been introduced. In another aspect the invention features a method for producing a binding molecule that binds human GITR, comprising culturing the host cell of the invention in a culture medium until a binding molecule that binds human GITR is produced by the cell.

### ***Brief Description of the Drawings***

10 ***Figure 1*** depicts an SDS-PAGE blot of purified mouse and human GITR binding molecules. Twelve micrograms of protein was loaded per well.

***Figure 2*** depicts a size exclusion chromatography (SE-HPLC) of the purified human GITR binding molecule. Fifty micrograms of protein was injected onto the SE-HPLC column at a flow rate of 0.6 ml/min. Purity of the binding molecules by SE-HPLC yielded a population of binding molecules in which 99.8 % was in monomeric form and 0.2% aggregates.

***Figure 3*** depicts a FACS analysis of L-M (mouse fibroblast) cells transfected with the GITR gene that were stained with 50  $\mu$ l of supernatant fluid from GITR-expressing hybridoma cells. The GITR binding molecule stained GITR-transfected cells but not the untransfected L-M cells.

***Figure 4*** depicts a FACS analysis demonstrating that GITR is primarily expressed on activated lymphocytes. The 6C8 binding molecules stains CD4+, CD8+, CD25+ lymphocytes and very weakly stains CD103+ cells.

25 ***Figure 5*** depicts a saturation curve of the binding of the 6C8 binding molecule which was assessed by titrating biotin-labeled 6C8 on CD3-activated lymphocytes.

***Figure 6*** is a graph showing that the 6C8 binding molecule is costimulatory to T lymphocytes which are stimulated with sub-optimal OKT3 (anti-CD3; 0.01  $\mu$ g/ml) and incubated with either anti-CD28, or anti-GITR. An isotype control (IgG2b) was also used.

30 ***Figures 7A and 7B*** are graphs demonstrating that the 6C8 binding molecule does not induce apoptosis. Lymphocytes were activated with PHA for 3 days prior to the addition of 10  $\mu$ g/ml of YTH655 (an anti-CD2 antibody known to induce apoptosis on activated lymphocytes; Friend, P., *et al.* (1987) *Transplant. Proc.* 19:4317), 6C8, or an isotype control (IgG2b). Apoptosis was measured by cell viability counts (A) and annexin V staining (B) and measuring apoptosis by flow cytometry.

***Figure 8*** is a graph demonstrating that the 6C8 binding molecule does not block a primary mixed lymphocyte reaction (MLR). Lymphocytes from allogenic

donors were mixed in the presence of TRX1 (anti-human CD4), 6C8 or MOPC (an isotype control for TRX1) at various concentrations. The cells were incubated for 3 days and pulsed with  $^3\text{H}$ -thymidine 18 hours before the cells were harvested and counted.

*Figure 9* is a graph demonstrating that the 6C8 binding molecule blocks the suppression of T effector cells induced by Treg cells. CD4+/CD25+ cells were added to CD4+/CD25- cells at various ratios. The cells were stimulated with plate-bound anti-CD3 and anti-CD28. At ratios of 1:1 there was inhibition of proliferation of the CD4+/CD25- cells. The addition of 6C8 at two different dilutions was able to block the suppression of CD4+ T effector cells induced by the CD4+/CD25+ T regulatory cells.

*Figure 10* is a graph demonstrating that the 6C8 binding molecule is co-stimulatory even when T cells are stimulated with anti-CD3 in the absence of anti-CD28. CD4+/CD25+ cells were incubated with CD4+/CD25- cells at different cell ratios. The cells were stimulated with plate-bound anti-CD3 only. 6C8 was added to the cells and under these conditions was co-stimulatory.

*Figure 11* is a graph demonstrating the effect of anti-GITR on I- $\kappa$ B degradation in CD3 activated T cells.

*Figure 12* is a graph demonstrating the effect of anti-GITR on I- $\kappa$ B phosphorylation in CD3 activated T cells.

*Figure 13* is a graph demonstrating the effect of anti-GITR on I- $\kappa$ B degradation, in CD3 plus CD28 activated T cells.

*Figure 14* is a graph demonstrating the effect of anti-GITR on I- $\kappa$ B phosphorylation, CD3 plus CD28 activated T cells.

*Figure 15* is a graph demonstrating that 6C8 and the R&D Systems (Minneapolis, MN) antibody recognize unique epitopes. The competition assay was performed on both OKT3 and Con A activated lymphocytes. One  $\mu\text{g/ml}$  of 6C8 was used with various amounts of the competing R&D Systems antibody (GITR/TNFRSF18 monoclonal antibody). There was some competition observed at the highest concentration of antibody, but this is most likely due to steric hindrance.

*Figure 16* shows the kinetic analysis of the 6C8 anti-GITR antibody versus the R&D Systems GITR antibody.

*Figure 17* is a graph showing the percent survival of mice injected with mitomycin C treated B16 cells following treatment with anti-GITR antibody (2F8 rat anti-mouse GITR binding molecule).

*Figures 18A-18D* depict the nucleic acid and amino acid sequences of the variable heavy chain (VHD) (A and B, respectively) and variable light chain (VKA) (C and D, respectively) of the 6C8 binding molecule. The leader sequences are shown in bold; the framework sequences are underlined; the CDR sequences are italicized.

*Figures 19A and 19B* are graphs showing that 2F8 and 2F8 F(ab')<sub>2</sub> fragments enhance the humoral response to HA.

*Figures 20A and 20B* are graphs showing that 2F8 and 2F8 F(ab')<sub>2</sub> fragments enhance the humoral response to Ova.

5

### ***Detailed Description of the Invention***

The present invention provides binding molecules that specifically bind to GITR, *e.g.*, human GITR (hGITR), on T cells and dendritic cells. The binding  
10 molecules of the invention are characterized by binding to hGITR with high affinity, and in the presence of a stimulating agent, *e.g.*, CD3, they are agonistic, and they abrogate the suppression of T effector (Teff) cells by T regulatory (Treg) cells. Various aspects of the invention relate to binding molecules, and pharmaceutical compositions thereof, as well as nucleic acids encoding binding molecules, recombinant expression vectors and  
15 host cells for making such binding molecules. Methods of using a binding molecule of the invention to detect human GITR or to modulate human GITR activity, either *in vitro* or *in vivo*, are also encompassed by the invention.

In order that the present invention may be more readily understood,  
20 certain terms are first defined.

### ***I. Definitions***

The term "glucocorticoid-induced TNF receptor" (abbreviated herein as "GITR"), also known as TNF receptor superfamily 18 (TNFRSF18), as used herein,  
25 refers to a member of the tumor necrosis factor/nerve growth factor receptor family. It is a 241 amino acid type I transmembrane protein characterized by three cysteine pseudorepeats in the extracellular domain and specifically protects T-cell receptor-induced apoptosis, although it does not protect cells from other apoptotic signals, including Fas triggering, dexamethasone treatment, or UV irradiation (Nocentini, G, *et al.* (1997) *Proc. Natl. Acad. Sci., USA* 94:6216-622). The nucleic acid sequence of  
30 human GITR (hGITR) is set forth in SEQ ID NO.: 17 and the amino acid sequence is set forth in SEQ ID NO.: 18.

The term "binding molecule" as used herein includes molecules that contain at least one antigen binding site that specifically binds to GITR. By "specifically  
35 binds" it is meant that the binding molecules exhibit essentially background binding to non-GITR molecules. An isolated binding molecule that specifically binds GITR may, however, have cross-reactivity to GITR molecules from other species.

The binding molecules of the invention may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Binding molecules may have both a heavy and a light chain. As used herein, the term binding molecule also includes, antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, so long as they exhibit the desired activity, *e.g.*, binding to GITR.

An "antigen" is an entity (*e.g.*, a proteinaceous entity or peptide) to which a binding molecule specifically binds.

The term "epitope" or "antigenic determinant" refers to a site on an antigen to which a binding molecule specifically binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, X-ray crystallography and 2-dimensional nuclear magnetic resonance. See, *e.g.*, *Epitope Mapping Protocols in Methods in Molecular Biology*, Vol. 66, G. E. Morris, Ed. (1996).

Binding molecules that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen, *i.e.*, a competitive binding assay. Competitive binding is determined in an assay in which the binding molecule being tested inhibits specific binding of a reference binding molecule to a common antigen, such as GITR. Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA); solid phase direct or indirect enzyme immunoassay (EIA) sandwich competition assay (see Stahli *et al.*, *Methods in Enzymology* 9:242 (1983)); solid phase direct biotin-avidin EIA (see Kirkland *et al.*, *J. Immunol.* 137:3614 (1986)); solid phase direct labeled assay, solid phase direct labeled sandwich assay (see Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press (1988)); solid phase direct label RIA using I-125 label (see Morel *et al.*, *Mol. Immunol.* 25(1):7 (1988)); solid phase direct biotin-avidin EIA (Cheung *et al.*, *Virology* 176:546 (1990)); and direct labeled RIA. (Moldenhauer *et al.*, *Scand. J. Immunol.* 32:77 (1990)).



Typically, such an assay involves the use of purified antigen bound to a solid surface or cells bearing either of these, an unlabeled test binding molecule and a labeled reference binding molecule. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test binding molecule.

5 Usually the test binding molecule is present in excess. Usually, when a competing binding molecule is present in excess, it will inhibit specific binding of a reference binding molecule to a common antigen by at least 50-55%, 55-60%, 60-65%, 65-70% 70-75% or more.

An epitope is also recognized by immunologic cells, for example, B cells  
10 and/or T cells. Cellular recognition of an epitope can be determined by *in vitro* assays that measure antigen-dependent proliferation, as determined by <sup>3</sup>H-thymidine incorporation, by cytokine secretion, by antibody secretion, or by antigen-dependent killing (cytotoxic T lymphocyte assay).

The term "monoclonal binding molecule" as used herein refers to a  
15 binding molecule obtained from a population of substantially homogeneous binding molecules. Monoclonal binding molecules are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal binding molecule preparations which typically include different binding molecules directed against different determinants (epitopes), each monoclonal binding molecule is directed against  
20 a single determinant on the antigen. The modifier "monoclonal" indicates the character of the binding molecule as being obtained from a substantially homogeneous population of binding molecules, and is not to be construed as requiring production of the binding molecule by any particular method. For example, the monoclonal binding molecules to be used in accordance with the present invention may be made by the hybridoma method  
25 first described by Kohler, *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Pat. No. 4,816,567). The "monoclonal binding molecules" may also be isolated from phage antibody libraries using the techniques described in Clackson, *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol Biol.* 222:581-597 (1991), for example.

30 The term "chimeric binding molecule" refers to a binding molecule comprising amino acid sequences derived from different species. Chimeric binding molecules can be constructed, for example by genetic engineering, from binding molecule gene segments belonging to different species.

The monoclonal binding molecules herein specifically include "chimeric"  
35 binding molecules in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in binding molecules derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in binding

molecules derived from another species or belonging to another antibody class or subclass, as well as fragments of such binding molecules, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison, *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). *e.g.*, binding to human GITR (hGITR).

5           Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VL) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3)  
10       confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The N-terminus is a variable region and at the C-terminus is a constant region; the CH3 and CL domains actually comprise the  
15       carboxy-terminus of the heavy and light chain, respectively.

          A "variable region" when used in reference to a binding molecule refers to the amino terminal portion of a binding molecule which confers antigen binding onto the molecule and which is not the constant region. The term includes functional fragments thereof which maintain some or all of the binding function of the whole  
20       variable region.

          The term "hypervariable region" when used herein refers to the regions of a binding molecule variable domain which are hypervariable in sequence and/or form structurally defined loops. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR".

25           As used herein, the term "CDR" or "complementarity determining region" means the noncontiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. These particular regions have been described by Kabat, *et al.*, *J. Biol. Chem.* 252, 6609-6616 (1977) and Kabat, *et al.*, *Sequences of protein of immunological interest.* (1991), and by Chothia, *et al.*, *J. Mol. Biol.* 196:901-917 (1987) and by MacCallum, *et al.*, *J. Mol. Biol.* 262:732-745 (1996)  
30       where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of a binding molecule or grafted binding molecule or variants thereof is within the scope of the term as defined and used herein.

35           As used herein, the term "framework region" or "FR" means each domain of the framework that is separated by the CDRs. Therefore, a variable region framework is between about 100-120 amino acids in length but refers only those amino acids outside of the CDRs.

"Humanized" forms of non-human (*e.g.*, murine) binding molecules are chimeric antibodies which contain minimal sequence derived from non-human binding molecule. For the most part, humanized binding molecules are human binding molecules (acceptor/recipient binding molecule) in which residues from a hyper-variable region are replaced by residues from a hypervariable region of a non-human species (donor binding molecule) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human binding molecule are altered, *e.g.*, replaced by, substituted, or backmutated to corresponding non-human residues. Furthermore, humanized binding molecules may comprise residues which are not found in the recipient binding molecule or in the donor binding molecule. These modifications are generally made to further refine binding molecule performance. In general, the humanized binding molecule will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human binding molecule and all or substantially all of the FR regions are those of a human binding molecule sequence. The humanized binding molecule optionally also will comprise at least a portion of a binding molecule constant region (Fc), typically that of a human binding molecule. For further details, see Jones, *et al.*, *Nature* 321:522-525 (1986); Riechmann, *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

Preferably, a humanized binding molecule of the invention comprises at least one CDR selected from the group consisting of SEQ ID NO.:3 (GFSLSTSGMGVG (HC CDR1)), SEQ ID NO.:4 (HIWWDDDKYYNPSLKS (HC CDR2N)), SEQ ID NO.:5 (TRRYFPFAY (HC CDR3)), SEQ ID NO.:6 (KASQNVGTNVA (LC CDR1)), SEQ ID NO.:7 (SASYRYS (LC CDR2)), SEQ ID NO.:8 (QQYNTDPLT (LC CDR3)), and SEQ ID NO.:19 (HIWWDDDKYYQPSLKS (HC CDR2Q)).

The term "engineered" or "recombinant" binding molecule, as used herein includes binding molecules that are prepared, expressed, created or isolated by recombinant means, such as binding molecules expressed using a recombinant expression vector transfected into a host cell, binding molecules isolated from a recombinant, combinatorial binding molecule library, binding molecules isolated from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see *e.g.*, Taylor, L.D., *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or binding molecules prepared, expressed, created or isolated by any other means that involves splicing of human binding molecule gene sequences to other DNA sequences. In certain embodiments, however, such recombinant human binding molecules are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions



of the recombinant binding molecules are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human binding molecule germline repertoire *in vivo*.

An "isolated binding molecule", as used herein, refers to a binding molecule that is substantially free of other binding molecules having different antigenic specificities (*e.g.*, an isolated binding molecule that specifically binds GITR is substantially free of binding molecules that specifically bind antigens other than GITR). Moreover, an isolated binding molecule may be substantially free of other cellular material and/or chemicals. An "isolated" binding molecule is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment include, *e.g.*, materials which would interfere with diagnostic or therapeutic uses for the binding molecule, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the binding molecule will be purified (1) to greater than 95% by weight of binding molecule as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated binding molecules include binding molecules *in situ* within recombinant cells since at least one component of the binding molecule's natural environment will not be present. Ordinarily, however, isolated binding molecules will be prepared by at least one purification step.

As used herein the term "binding constant" "(kd)", also referred to as "affinity constant", is a measure of the extent of a reversible association between two molecular species includes both the actual binding affinity as well as the apparent binding affinity. The actual binding affinity is determined by calculating the ratio of the  $K_{\text{assoc}}$  in  $M^{-1}S^{-1}$  to the  $K_{\text{dissoc}}$  in  $S^{-1}$  and has the units " $M^{-1}$ ". Therefore, conferring or optimizing binding affinity includes altering either or both of these components to achieve the desired level of binding affinity. The apparent affinity can include, for example, the avidity of the interaction. For example, a bivalent heteromeric variable region binding fragment can exhibit altered or optimized binding affinity due to its valency. Binding affinity can be determined by measurement of surface plasmon resonance, *e.g.*, using a BLAcore system.

The term "nucleic acid molecule", as used herein, includes DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule", as used herein in reference to nucleic acids encoding binding molecules that bind GITR, refers to a nucleic acid



molecule in which the nucleotide sequences encoding the binding molecule are free of other nucleotide sequences which other sequences may naturally flank the nucleic acid in human genomic DNA. These sequences may optionally include 5' or 3' nucleotide sequences important for regulation or protein stability.

5           The term "vector", as used herein, refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors  
10 are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the  
15 expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However,  
20 the invention includes such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

          The term "recombinant host cell" (or simply "host cell"), as used herein, refers to a cell into which a recombinant expression vector has been introduced. It  
25 should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

30           As used herein, the term "T cell" (*i.e.*, T lymphocyte) includes all cells within the T cell lineage, including thymocytes, immature T cells, mature T cells and the like, from a mammal (*e.g.*, human). Preferably, T cells are mature T cells that express either CD4 or CD8, but not both, and a T cell receptor. The various T cell populations described herein can be defined based on their cytokine profiles and their function, and  
35 are known to one of skill in the art.

          As used herein, the term "dendritic cell" refers to professional antigen-presenting cells (APCs) capable of activating naïve T cells and stimulating the growth and differentiation of B cells.

As used herein, the term "naïve T cells" includes T cells that have not been exposed to cognate antigen and so are not activated or memory cells. Naïve T cells are not cycling and human naïve T cells are CD45RA+. If naïve T cells recognize antigen and receive additional signals depending upon but not limited to the amount of antigen, route of administration and timing of administration, they may proliferate and differentiate into various subsets of T cells, *e.g.* effector T cells.

As used herein, the term "effector T cell" or "Teff cell" includes T cells which function to eliminate antigen (*e.g.*, by producing cytokines which modulate the activation of other cells or by cytotoxic activity). The term "effector T cell" includes T helper cells (*e.g.*, Th1 and Th2 cells) and cytotoxic T cells. Th1 cells mediate delayed type hypersensitivity responses and macrophage activation while Th2 cells provide help to B cells and are critical in the allergic response (Mosmann and Coffman, 1989, *Annu. Rev. Immunol.* 7, 145-173; Paul and Seder, 1994, *Cell* 76, 241-251; Arthur and Mason, 1986, *J. Exp. Med.* 163, 774-786; Paliard, *et al.*, 1988, *J. Immunol.* 141, 849-855; Finkelman, *et al.*, 1988, *J. Immunol.* 141, 2335-2341).

As used herein, the term "T helper type 1 response" (Th1 response) refers to a response that is characterized by the production of one or more cytokines selected from IFN- $\gamma$ , IL-2, TNF, and lymphotoxin (LT) and other cytokines produced preferentially or exclusively by Th1 cells rather than by Th2 cells. As used herein, a "T helper type 2 response" (Th2 response) refers to a response by CD4+ T cells that is characterized by the production of one or more cytokines selected from IL-4, IL-5, IL-6 and IL-10, and that is associated with efficient B cell "help" provided by the Th2 cells (*e.g.*, enhanced IgG1 and/or IgE production).

As used herein, the term "regulatory T cell" or "Treg cell" includes T cells which produce low levels of IL-2, IL-4, IL-5, and IL-12. Regulatory T cells produce TNF $\alpha$ , TGF $\beta$ , IFN- $\gamma$ , and IL-10, albeit at lower levels than effector T cells. Although TGF $\beta$  is the predominant cytokine produced by regulatory T cells, the cytokine is produced at levels less than or equal to that produced by Th1 or Th2 cells, *e.g.*, an order of magnitude less than in Th1 or Th2 cells. Regulatory T cells can be found in the CD4+CD25+ population of cells (see, *e.g.*, Waldmann and Cobbold. 2001. *Immunity.* 14:399). Regulatory T cells actively suppress the proliferation and cytokine production of Th1, Th2, or naïve T cells which have been stimulated in culture with an activating signal (*e.g.*, antigen and antigen presenting cells or with a signal that mimics antigen in the context of MHC, *e.g.*, anti-CD3 antibody, plus anti-CD28 antibody).

As used herein, the term "tolerance" includes refractivity to activating receptor-mediated stimulation. Such refractivity is generally antigen-specific and persists after exposure to the tolerizing antigen has ceased. For example, tolerance is characterized by lack of cytokine production, *e.g.*, IL-2, or can be assessed by use of a

mixed lymphocyte culture assay. Tolerance can occur to self antigens or to foreign antigens.

A "mixed lymphocyte culture" ("MLC") is a type of lymphocyte proliferation test in which lymphocytes from two individuals are cultured together and the proliferative response ("mixed lymphocyte reaction") is measured by <sup>3</sup>H-labeled thymidine uptake.

As used herein, the term "apoptosis" also referred to as programmed cell death (PCD), is the death of a cell characterized by features including, but not limited to, condensation of nuclear heterochromatin, cell shrinkage, cytoplasmic condensation, and in a later stage of apoptosis, endonuclease mediated cleavage of the DNA of the cell into discrete fragments. Upon electrophoretic analysis of the DNA of a cell in which apoptosis has occurred, a characteristic "ladder" of discrete DNA fragments may be apparent.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment may include those already having a disorder as well as those which do not yet have a disorder.

A "disorder" is any condition that would benefit from treatment with a binding molecule of the present invention. This includes chronic and acute disorders or diseases or pathological conditions associated with immune responses that are too high or too low.

Various aspects of the invention are described in further detail in the following subsections.

## 25 **II. *GITR Binding molecules***

The present invention provides isolated GITR binding molecules. Exemplary binding molecules of the present invention include the 6C8 antibody and the 2F8 antibody. The 6C8 antibody is an anti-GITR antibody that binds to GITR on T cells and dendritic cells, *e.g.*, human T cells and dendritic cells, with high affinity. Preferably, such binding molecules abrogate the suppression of Teff cells by Treg cells and are agonistic to partially activated T cells *in vitro* in the presence of a stimulating agent, *e.g.*, CD3.

In one embodiment, the a VH domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:1. (6C8 VH domain "N", including leader). It will be understood that although some of the sequences of binding molecules described herein include leader sequences, a binding molecule of the invention may also exclude the leader sequence, which is optional. For example, in one embodiment, a binding molecule of the invention comprises the amino

acid sequence of the mature protein shown in SEQ ID NO:1. e.g., amino acids 20-138 of SEQ ID NO:1.

In one embodiment, the a VH domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:66. (6C8 VH domain “Q”, including leader).

In one embodiment, the a VL domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:2. (6C8 VL domain, including leader).

In one embodiment, the a VH domain of a binding molecule of the invention comprises amino acid residues 20-138 of SEQ ID NO.:1. (6C8 VH domain “N”, without leader).

In one embodiment, the a VH domain of a binding molecule of the invention comprises amino acid residues 20-138 of SEQ ID NO.:66. (6C8 VH domain “Q”, without leader).

In one embodiment, the a VL domain of a binding molecule of the invention comprises comprises amino acid residues 21-127 of SEQ ID NO.:2. (6C8 VL domain, without leader).

In one embodiment of the invention the VL chain comprises a leader and/or signal sequence, i.e., amino acid residues 1-20 of SEQ ID NO:2 (SEQ ID NO:59). In one embodiment, the VH chain comprises a leader and/or signal sequence, i.e., amino acid residues 1-19 of SEQ ID NO:1 (SEQ ID NO:64).

In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR set forth in SEQ ID NO:3. (6C8 VH CDR1).

In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR set forth in SEQ ID NO:4. (6C8 VH CDR2-“N”).

In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR set forth in SEQ ID NO:5. (6C8 VH CDR3).

In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR set forth in SEQ ID NO:19. (6C8 VH CDR2-alternate “Q”).

In one embodiment, a binding molecule of the invention comprises a VL domain comprising a CDR set forth in SEQ ID NO:6. (6C8 VL CDR1).

In one embodiment, a binding molecule of the invention comprises a VL domain comprising a CDR set forth in SEQ ID NO:7. (6C8 VL CDR2).

In one embodiment, a binding molecule of the invention comprises a VL domain comprising a CDR set forth in SEQ ID NO:8. (6C8 VL CDR3).

The invention also pertains to nucleic acid molecules encoding the above amino acid sequences.



In one embodiment, the a VH domain of a binding molecule of the invention comprises the nucleotide sequence set forth in SEQ ID NO:9. (6C8 VH domain, "N", including leader).

5 In one embodiment, the a VH domain of a binding molecule of the invention comprises the nucleotide sequence set forth in SEQ ID NO:65. (6C8 VH domain, "Q", including leader).

In one embodiment, the a VH domain of a binding molecule of the invention comprises nucleotides 58-414 of SEQ ID NO.:9. (6C8 VH domain, "N", without leader).

10 In one embodiment, the a VH domain of a binding molecule of the invention comprises nucleotides 58-414 of SEQ ID NO.:65. (6C8 VH domain, "Q", without leader).

In one embodiment, the a VL domain of a binding molecule of the invention comprises the nucleotide sequence set forth in SEQ ID NO:10. (6C8 VL domain, including leader).

In one embodiment, the a VL domain of a binding molecule of the invention comprises nucleotides 61-381 of SEQ ID NO.:10. (6C8 VL domain, without leader).

20 In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR the nucleic acid sequence of which is set forth in SEQ ID NO:11. (6C8 VH CDR1).

In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR the nucleic acid sequence of which is set forth in SEQ ID NO:12. (6C8 VH CDR2-"AAT").

25 In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR the nucleic acid sequence of which is set forth in SEQ ID NO:13. (6C8 VH CDR3).

In one embodiment, a binding molecule of the invention comprises a VH domain comprising a CDR the nucleic acid sequence of which is set forth in SEQ ID NO:65. (6C8 VH CDR2-alternate "CAA").

30 In one embodiment, a binding molecule of the invention comprises a VL domain comprising a CDR the nucleic acid sequence of which is set forth in SEQ ID NO:14. (6C8 VL CDR1).

In one embodiment, a binding molecule of the invention comprises a VL domain comprising a CDR the nucleic acid sequence of which is set forth in SEQ ID NO:15. (6C8 VL CDR2).

In one embodiment, a binding molecule of the invention comprises a VL domain comprising a CDR the nucleic acid sequence of which is set forth in SEQ ID NO:16. (6C8 VL CDR3).

5 In one embodiment, the a CL domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:20. (Murine IgG2a light chain constant region).

In one embodiment, the a CH domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:21. (Murine IgG2a heavy chain constant region).

10 In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:22. (Chimeric-6C8 VL/human CL IgG1).

In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:23. (Chimeric Gly-6C8 VH/human CH IgG1).

15 In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:24. (Chimeric Agly-6C8 VH/human CH IgG1).

In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:44. (Humanized 6C8 VL).

20 In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:53. (Humanized 6C8 VH "N").

In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:54. (Humanized 6C8 VH "Q").

25 In one embodiment, the a CL domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:55. (Human IgG1 Gly heavy chain constant region).

In one embodiment, the a CH domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:56. (Human IgG1 Agly heavy chain constant region).

30 In one embodiment, the a CL domain of a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:57. (Human IgG1 light chain constant region).

In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:58. (Complete Humanized 6C8 Light).

35 In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:60. (Complete Humanized 6C8 Heavy-HuN6C8-Gly).

In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:61. (Complete Humanized 6C8 Heavy-HuN6C8-Agly).

In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:62. (Complete Humanized 6C8 Heavy-HuQ6C8-Gly).

In one embodiment, a binding molecule of the invention comprises the amino acid sequence set forth in SEQ ID NO:63. (Complete Humanized 6C8 Heavy-HuQ6C8-Agly).

In one embodiment, a binding molecule of the invention has VL and VH sequences as shown in Figures 18A-18D; the amino acid sequence of the 6C8 VH region is also shown in SEQ ID NO: 1; the amino acid sequence of the 6C8 VL region is shown in SEQ ID NO: 2. In another embodiment, a binding molecule of the invention has LC and HC sequences as set forth in SEQ ID NOs:20 and 21;

ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSRQNGVLNS  
WTDQDSKDYSTYSMSSTLTTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNE (SEQ  
ID NO:20);

AKTTPPSVYPLAPGCGDTTGSSVTLGCLVKGYFPESVTVTWNSGSLSSSVHTFPA  
LLQSGLYTMSSSVTVPSSTWPSQTVTCSVAHPASSTTVDDKKLEPSGPSTINPCPP  
CKECKCPAPNLEGGPSVFIFPPNIKDVLMLSLTPKVTCTVVDVSEDDPDVQISWF  
VNNVEVHTAQTQTHREDYNSTIRVVSTLPIQHQDWMSGKEFKCKVNNKDLPSPI  
ERTISKIKGLVRAQVYILPPPAEQLSRKDVSLTCLVVGFNPGDISVEWTSNGHTE  
ENYKDTAPVLDSGYSYFIYSKLNMKTSKWKTDSFSCNVRHEGLKNYYLKKKTIS  
RSPGK (SEQ ID NO:21).

In one embodiment of the invention the VL chain comprises a leader and/or signal sequence, *e.g.*, amino acid residues 1-20 of SEQ ID NO:2. In one embodiment, the VH chain comprises a leader and/or signal sequence, *e.g.*, amino acid residues 1-19 of SEQ ID NO:1. In another embodiment, a binding molecule of the invention does not comprise a leader and/or signal sequence.

In one aspect, the invention pertains to 6C8 binding molecules and other binding molecules with equivalent properties to 6C8, such as high affinity binding to GITR and abrogation of suppression of Teff cells by Treg cells. In addition, the binding molecules of the invention do not induce apoptosis, nor do they inhibit a mixed lymphocyte reaction. Accordingly, equivalent binding molecules of the invention are GITR agonists, *i.e.*, they induce signaling *via* GITR. GITR is a member of the TNFR superfamily. Since members of the TNFR family are involved in cell survival and apoptosis by signaling through NF- $\kappa$ B, in one embodiment, the binding molecules of the present invention attenuate degradation of I- $\kappa$ B.

In one embodiment, the invention provides isolated hGTR binding molecules with a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 2, and optionally a leader sequence, and a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO: 1, and optionally a leader sequence. In certain embodiments, a binding molecule comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Furthermore, the binding molecule can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the binding molecule comprises a kappa light chain constant region. In one embodiment, a binding molecule of the invention comprises a light chain constant region as set forth in SEQ ID NO:20. In one embodiment, a binding molecule of the invention comprises a heavy chain constant region as set forth in SEQ ID NO:21. In one embodiment, a binding molecule of the invention comprises a heavy chain constant region as set forth in SEQ ID NO:55. In one embodiment, a binding molecule of the invention comprises a heavy chain constant region as set forth in SEQ ID NO:56. In one embodiment, a binding molecule of the invention comprises a heavy chain constant region as set forth in SEQ ID NO:57.

In another embodiment, the invention provides a binding molecule having 6C8-related VL CDR domains, for example, binding molecules with a light chain variable region (VL) having at least one CDR domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8. In another embodiment, a light chain variable region (VL) has at least two CDR domains comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8. In yet another embodiment, a light chain variable region (VL) has CDR domains comprising the amino acid sequences consisting of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8.

In still other embodiments, the invention provides a binding molecule having 6C8-related VH CDR domains, for example, binding molecules with a light chain variable region (VH) having a CDR domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO:19. In another embodiment, a heavy chain variable region (VH) has at least two CDR domains comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO:19. In yet another embodiment, a heavy chain variable region (VH) has CDR domains comprising the amino acid sequences consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO:19.

In another embodiment, a binding molecule of the invention comprises at least one CDR derived from a murine anti-human GTR binding molecule, *e.g.*, a 6C8



binding molecule. As used herein the term “derived from” a designated protein refers to the origin of the polypeptide. In one embodiment, the polypeptide or amino acid sequence which is derived from a particular starting polypeptide is a CDR sequence or sequence related thereto. In another embodiment, the polypeptide or amino acid  
5 sequence which is derived from a particular starting polypeptide is a FR sequence or sequence related thereto. In one embodiment, the amino acid sequence which is derived from a particular starting polypeptide is not contiguous.

For example, in one embodiment, one, two, three, four, five, or six CDRs are derived from a murine 6C8 antibody. In one embodiment, a binding molecule of the  
10 invention comprises at least one heavy or light chain CDR of a murine 6C8 antibody. In another embodiment, a binding molecule of the invention comprises at least two CDRs from a murine 6C8 antibody. In another embodiment, a binding molecule of the invention comprises at least three CDRs from a murine 6C8 antibody. In another  
15 embodiment, a binding molecule of the invention comprises at least four CDRs from a murine 6C8 antibody. In another embodiment, a binding molecule of the invention comprises at least five CDRs from a murine 6C8 antibody. In another embodiment, a binding molecule of the invention comprises at least six CDRs from a murine 6C8 antibody.

It will also be understood by one of ordinary skill in the art that a binding  
20 molecule of the invention may be modified such that they vary in amino acid sequence from the 6C8 molecule from which they were derived. For example, nucleotide or amino acid substitutions leading to conservative substitutions or changes at “non-essential” amino acid residues may be made (*e.g.*, in CDR and/or framework residues) and maintain the ability to bind to GITR, *e.g.*, human GITR.

In one embodiment, the at least one CDR (or at least one CDR from the  
25 greater than one 6C8 CDRs that are present in the binding molecule) is modified to vary in sequence from the CDR of a naturally occurring 6C8 binding molecule, yet retains the ability to bind to 6C8. For example, in one embodiment, one or more CDRs from a 6C8 antibody are modified to remove potential glycosylation sites. For example, since the  
30 amino acid sequence Asn-X- (Ser/Thr) is a putative consensus sequence for a glycosylation site which may affect the production of the binding molecule, and CDR2 of the 6C8 heavy chain has the sequence Asn- Pro-Ser, a second version of the heavy chain was prepared to conservatively substitute a glutamine (Gln) for an asparagine (Asn) at amino acid residue 62 of SEQ ID NO:53.

In one embodiment, a binding molecule of the invention comprises a  
35 polypeptide or amino acid sequence that is essentially identical to that of a 6C8 antibody, or a portion thereof wherein the portion consists of at least 3-5 amino acids, of at least 5-10 amino acids, at least 10-20 amino acids, at least 20-30 amino acids, or at

least 30-50 amino acids, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the starting sequence.

In another embodiment, the polypeptide or amino acid sequence which is derived from a particular starting polypeptide or amino acid sequence shares an amino acid sequence identity that is about 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the starting sequence.

An isolated nucleic acid molecule encoding a non-natural variant of a polypeptide can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of the binding molecule such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. In one embodiment, conservative amino acid substitutions are made at one or more non-essential amino acid residues. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in a binding molecule polypeptide may be replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members.

Alternatively, in another embodiment, mutations may be introduced randomly along all or part of the binding molecule coding sequence.

Preferred binding molecules of the invention comprise framework and constant region amino acid sequences derived from a human amino acid sequence. However, binding molecules may comprise framework and/or constant region sequences derived from another mammalian species. For example, a primate framework region (*e.g.*, non-human primate), heavy chain portion, and/or hinge portion may be included in the subject binding molecules. In one embodiment, one or more murine amino acids may be present in the framework region of a binding polypeptide, *e.g.*, a human or non-human primate framework amino acid sequence may comprise one or more amino acid substitutions and/or backmutations in which the corresponding murine amino acid

residue is present. Preferred binding molecules of the invention are less immunogenic than the starting 6C8 murine antibody.

The present invention also features chimeric and/or humanized binding molecules (*i.e.*, chimeric and/or humanized immunoglobulins) specific for GITR.

5 Chimeric and/or humanized binding molecules have the same or similar binding specificity and affinity as a mouse or other nonhuman binding molecules that provide the starting material for construction of a chimeric or humanized binding molecule.

A chimeric binding molecule is one whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the  
10 genes from a mouse monoclonal binding molecule may be joined to human constant (C) segments, such as IgG1 or IgG4. Human isotype IgG1 is preferred. An exemplary chimeric binding molecule is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse binding molecule and the C or effector domain from a  
15 human binding molecule.

In one embodiment, the invention pertains to humanized variable regions of the 6C8 binding molecule and polypeptides comprising such humanized variable regions. In one embodiment, a binding molecule of the invention comprises at least one humanized 6C8 binding molecule variable region, *e.g.*, a light chain or heavy chain  
20 variable region.

The term "humanized binding molecule" refers to a binding molecule comprising at least one chain comprising variable region framework residues derived from a human binding molecule chain (referred to as the acceptor immunoglobulin or binding molecule) and at least one complementarity determining region derived from a  
25 mouse-binding molecule, (referred to as the donor immunoglobulin or binding molecule). Humanized binding molecules can be produced using recombinant DNA technology, which is discussed below. See for example, *e.g.*, Hwang, W.Y.K., *et al.* (2005) *Methods* 36:35; Queen *et al.*, *Proc. Natl. Acad. Sci. USA*, (1989), 86:10029-10033; Jones *et al.*, *Nature*, (1986), 321:522-25; Riechmann *et al.*, *Nature*, (1988),  
30 332:323-27; Verhoeven *et al.*, *Science*, (1988), 239:1534-36; Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA*, (1989), 86:3833-37; US Patent Nos. US 5,225,539; 5,530,101; 5,585,089; 5,693,761; 5,693,762; 6,180,370, Seltick *et al.*, WO 90/07861, and Winter, US 5,225,539 (incorporated by reference in their entirety for all purposes). The constant region(s), if present, are preferably is also derived from a human immunoglobulin.

35 When a preferred non-human donor binding molecule has been selected for humanization, an appropriate human acceptor binding molecule may be obtained, *e.g.*, from sequence databases of expressed human antibody genes, from germline Ig sequences or a consensus sequence of several human binding molecules.



In one embodiment, a CDR homology based method is used for humanization (see, *e.g.*, Hwang, W.Y.K., *et al.* (2005) *Methods* 36:35, the contents of which is incorporated in its entirety herein by this reference). This method generally involves substitution of mouse CDRs into a human variable domain framework based on similarly structured mouse and human CDRs rather than similarly structured mouse and human frameworks. The similarity of the mouse and human CDRs is generally determined by identifying human genes of the same chain type (light or heavy) that have the same combination of canonical CDR structures as the mouse binding molecules and thus retain three-dimensional conformation of CDR peptide backbones. Secondly, for each of the candidate variable genes with matching canonical structures, residue to residue homology between the mouse and candidate human CDRs is evaluated. Finally, to generate a humanized binding molecule, CDR residues of the chosen human candidate CDR not already identical to the mouse CDR are converted to the mouse sequence. In one embodiment, no mutations of the human framework are introduced into the humanized binding molecule.

In one embodiment, human germline sequences are evaluated for CDR homology to the GITR binding molecule CDRs. For example, for the murine 6C8 antibody, all germ line light chain kappa chain V genes with a 2-1-1 canonical structure in the IMGT database were compared with the 6C8 antibody sequence. The same was done for the heavy chain where all 3-1 germ line heavy chain V genes were compared to the 6C8 amino acid sequence. Accordingly, in one embodiment, a binding molecule of the invention comprises a human kappa chain V region framework with a 2-1-1 canonical structure. In another embodiment, a binding molecule of the invention comprises a human heavy chain V region framework with a 3-1 canonical structure.

The following potential human light chain germline sequences were identified and may be incorporated into a binding molecule of the invention:

The IMGT accession number of the IGKV3-15 gene is M23090. The amino acid sequence is:

EIVMTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTR  
ATGIPARFSGSGSGTEFTLTISSLQSEDAVYYCQQYNNWP (SEQ ID NO:25).

The IMGT accession number of the IGKV3D-11 gene is X17264. The amino acid sequence is:

EIVLTQSPATLSLSPGERATLSCRASQGVSSYLAWYQQKPGQAPRLLIYDASNRA  
TGIPARFSGSGPGTDFTLTISLLEPEDFAVYYCQQRSNWH (SEQ ID NO:26).

There are two alleles of the IGKV3-11 gene. The IMGT accession number of allele \*01 of the IGKV3-11 gene is X01668. The amino acid sequence is:  
EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRA  
TGIPARFSGSGSGTDFTLTISLLEPEDFAVYYCQQRSNWP (SEQ ID NO:27).



The IMGT accession number of allele \*02 of the IGKV3-11 gene is K02768. The amino acid sequence is:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRA  
TGIPARFSGSGSGRDFTLTISSLEPEDFAVYYCQQRSNWP (SEQ ID NO:28).

5 The IMGT accession number of the IGKV1D-43 gene is X72817. The amino acid sequence is:

AIRMTQSPFSLASVGDRVITTCWASQGISSYLAWYQQKPAKAPKLFIIYASSLQ  
SGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQYYSTP (SEQ ID NO:29).

There are two alleles of the IGKV1-39 gene. The IMGT accession  
10 number of allele \*01 of the IGKV1-39 gene is X59315. The amino acid sequence is:  
DIQMTQSPSSLSASVGDRVITTCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQ  
SGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQSYSTP (SEQ ID NO:30).

The IMGT accession number of allele \*02 of the IGKV1-39 gene is X59318. The amino acid sequence is:

15 DIQMTQSPSFLASVGDRVITTCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQ  
SGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQCGYSTP (SEQ ID NO:31).

The IMGT accession number of the IGKV1-33 gene is M64856. The amino acid sequence is:

20 DIQMTQSPSSLSASVGDRVITTCQASQDISNYLNWYQQKPGKAPKLLIYDASNLE  
TGVPSRFSGSGSGTDFTFTISLQPEDATYYCQQYDNLP (SEQ ID NO:32).

The IMGT accession number of the IGKV1-27 gene is X63398. The amino acid sequence is:

DIQMTQSPSSLSASVGDRVITTCRASQGISNYLAWYQQKPGKVPKLLIYAASLQ  
SGVPSRFSGSGSGTDFTLTISLQPEDVATYYCQKYNSAP (SEQ ID NO:33).

25 There are two alleles of the IGKV1-17 gene. The IMGT accession number of allele \*01 of the IGKV1-17 gene is X72808. The amino acid sequence is:  
DIQMTQSPSSLSASVGDRVITTCRASQGIRNDLGWYQQKPGKAPKRLIYAASSL  
QSGVPSRFSGSGSGTEFTLTISLQPEDFATYYCLQHNSYP (SEQ ID NO:34).

The IMGT accession number of allele \*02 of the IGKV1-17 gene is D88255. The amino acid sequence is:

30 DIQMTQSPSSLSASVGDRVITTCRASQGIRNDLGWYQQKPGKAPKRLIYAASSL  
QSGVPSRFSGSGSGTEFTLTISNLQPEDFATYYCLQHNSYP (SEQ ID NO:35).

There are two alleles of the IGKV1D-16 gene. The IMGT accession number of allele \*01 of the IGKV1D-16 gene is K01323. The amino acid sequence is:

35 DIQMTQSPSSLSASVGDRVITTCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQ  
SGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYNSYP (SEQ ID NO:36).

The IMGT accession number of allele \*02 of the IGKV1D-16 gene is J00244. The amino acid sequence is:

DIQMTQSPSSLSASVGDRVTITCRARQGISSWLAWYQQKPEKAPKSLIYAASSLQ  
SGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYNLYP (SEQ ID NO:37).

The IMGT accession number of the IGKV1-16 gene is J00248. The amino acid sequence is:

5 DIQMTQSPSSLSASVGDRVTITCRASQGISNYLAWFQQKPGKAPKSLIYAASSLQ  
SGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYNLYP (SEQ ID NO:38).

There are two alleles of the IGKV1-12 gene. The IMGT accession number of allele \*01 of the IGKV1-12 gene is V01577. The amino acid sequence is:

DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYAASSL  
10 QSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQANLFP (SEQ ID NO:39).

The IMGT accession number of allele \*02 of the IGKV1-12 gene is V01576. The amino acid sequence is:

DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYAASSL  
QSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQANLFP (SEQ ID NO:40).

15 The IMGT accession number of the IGKV1-9 gene is Z00013. The amino acid sequence is:

DIQLTQSPSFLSASVGDRVTITCRASQGISSYLAWYQQKPGKAPKLLIYAASLQ  
SGVPSRFSGSGSGTEFTLTISLQPEDFATYYCQQNLNLYP (SEQ ID NO:41).

The IMGT accession number of the IGKV1-6 gene is M64858. The amino acid sequence is:

20 AIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKLLIYAASSLQ  
SGVPSRFSGSGSGTDFTLTISLQPEDFATYYCLQDYNLYP (SEQ ID NO:42).

There are three alleles of the IGKV1-5 gene. The IMGT accession number of allele \*01 of the IGKV1-5 gene is Z00001. The amino acid sequence is:

25 DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQQKPGKAPKLLIYDASSLE  
SGVPSRFSGSGSGTEFTLTISLQPDDEFATYYCQQYNLYS (SEQ ID NO:43).

The following potential human heavy chain germline sequences were identified and may be incorporated into a binding molecule of the invention:

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There are ten alleles of the IGHV2-5 gene. The IMGT accession number of allele \*01 of the IGHV2-5 gene is X62111. The amino acid sequence is:

QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALEWLALIYW  
NDDKRYSPSLKSRLTITKDTSKNQVVLTMNMDPVDATYY (SEQ ID NO:45).

35 The IMGT accession number of the IGHV2-26 gene is M99648. The amino acid sequence is:

QVTLKESGPVLVKPTETLTLTCTVSGFSLSNARMGVSWIRQPPGKALEWLAHIFS

NDEKSYSTSLKSRLTISKDTSKSQVVLTMTNMDPVDATATYYCARI (SEQ ID NO:46).

There are thirteen alleles of the IGHV2-70 gene. The IMGT accession number of allele \*01 of the IGHV2-70 gene is L21969. The amino acid sequence is:

5 QVTLRSGPALVKPTQTLTLTCTFSGFSLSTSGMCMVSWIRQPPGKALEWLALID  
WDDDKYYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDATATYYCARI (SEQ ID NO:47).

There are four alleles of the IGHV4-30-2 gene. The IMGT accession number of allele \*01 of the IGHV4-30-2 gene is L10089. The amino acid sequence is:

10 QLQLQESGSGLVKPSQTLTLTCAVSGGSISSGGYSWSWIRQPPGKGLEWIGYIYH  
SGSTYYNPSLKSRTISVDRSKNQFSLKLSSVTAADTAVYYCAR (SEQ ID NO:48).

There are six alleles of the IGHV4-30-4 gene. The IMGT accession number of allele \*01 of the IGHV4-30-4 gene is Z14238. The amino acid sequence is:

15 QVQLQESGPGLVKPSQTLTLTCTVSGGSISSGDYYWSWIRQPPGKGLEWIGYIYY  
SGSTYYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCAR (SEQ ID NO:49).

There are ten alleles of the IGHV4-31 gene. The IMGT accession number of allele \*01 of the IGHV2-5 gene is L10098. The amino acid sequence is:

20 QVQLQESGPGLVKPSQTLTLTCTVSGGSISSGGYYWSWIRQHPPGKGLEWIGYIY  
YSGSTYYNPSLKSLVTISVDTSKNQFSLKLSSVTAADTAVYYCAR (SEQ ID NO:50).

There are six alleles of the IGHV4-39 gene. The IMGT accession number of allele \*01 of the IGHV4-39 gene is L10094. The amino acid sequence is:

25 QLQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQPPGKGLEWIGSIYY  
SGSTYYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCAR (SEQ ID NO:51).

There are eight alleles of the IGHV4-61 gene. The IMGT accession number of allele \*01 of the IGHV4-61 gene is M29811. The amino acid sequence is:

30 QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGSYYWSWIRQPPGKGLEWIGYIY  
YSGSTNYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCAR (SEQ ID NO:52).

Each of these germline sequences may be used to provide framework regions for use with one or more 6C8 CDRs.

As used herein, "canonical structures" are conserved hypervariable loop conformations made by different CDRs by which the binding molecule forms the antigen contacts. The assignment of canonical structure classes to a new binding molecule can be achieved using publicly available software.

In another embodiment, the substitution of mouse CDRs into a human variable domain framework is based on the retention of the correct spatial orientation of the mouse variable domain framework by identifying human variable domain frameworks which will retain the same conformation as the mouse variable domain frameworks from which the CDRs were derived. In one embodiment, this is achieved by obtaining the human variable domains from human binding molecules whose framework sequences exhibit a high degree of sequence identity with the murine variable framework domains from which the CDRs were derived. See Kettleborough *et al.*, *Protein Engineering* 4:773 (1991); Kolbinger *et al.*, *Protein Engineering* 6:971 (1993) and Carter *et al.*, WO 92/22653.

Preferably the human acceptor binding molecule retains the canonical and interface residues of the donor binding molecule. Additionally, the human acceptor binding molecule preferably has substantial similarity in the length of CDR loops. See Kettleborough *et al.*, *Protein Engineering* 4:773 (1991); Kolbinger *et al.*, *Protein Engineering* 6:971 (1993) and Carter *et al.*, WO 92/22653.

In another embodiment, appropriate human acceptor sequences may be selected based on homology to framework regions of the 6C8 binding molecule. For example, the amino acid sequence of the 6C8 binding molecule may be compared to the amino acid sequence of other known binding molecules by, for example, by comparing the FR regions or the variable region sequences of the 6C8 amino acid sequence against a publicly available database of known binding molecules and selecting those sequences with the highest percent identity of amino acids in the variable or FR region, *i.e.*, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%. In one embodiment, the framework sequence set forth in SEQ ID NO:67 may be used

**(QVTLKESGPGILQPSQTLSTCSFSGFSLSTSGMGVGVWIRQPSGKGLEWLAH**  
**IWWDDDDKYNPSLKSRLTISKDTSSNQVFLKITSVDTRDTATYYCARTRRYFPF**  
**AYWGEGTSVTVT** (SEQ ID NO:67; Framework residues are in bold)). In another embodiment, the framework sequence set forth in SEQ ID NO:68 may be used

**(QVTLRESGPALVKPTQTLTLCTFSGFSLSTSGMGVGVWIRQPPGKALEWLA**  
**HIWWDDDDKYNPSLKSRLTISKDTSKNQVVLMTNMDPVDATYYCARTRRY**  
**FPFAYWGQGTLVTVSS** (SEQ ID NO:68; Framework residues are in bold)).

Having identified the complementarity determining regions of the murine donor immunoglobulin and appropriate human acceptor immunoglobulins, the next step is to determine which, if any, residues from these components should be substituted to optimize the properties of the resulting humanized binding molecule. In general, substitution of human amino acid residues with murine should be minimized, because introduction of murine residues increases the risk of the binding molecule eliciting a



human-anti-mouse-antibody (HAMA) response in humans. Art-recognized methods of determining immune response can be performed to monitor a HAMA response in a particular patient or during clinical trials. Patients administered humanized binding molecules can be given an immunogenicity assessment at the beginning and throughout  
5 the administration of said therapy. The HAMA response is measured, for example, by detecting antibodies to the humanized therapeutic reagent, in serum samples from the patient using a method known to one in the art, including surface plasmon resonance technology (BIAcore) and/or solid-phase ELISA analysis.

When necessary, one or more residues in the human framework regions  
10 can be changed or substituted to residues at the corresponding positions in the murine antibody so as to preserve the binding affinity of the humanized antibody to the antigen. This change is sometimes called "backmutation". Certain amino acids from the human variable region framework residues are selected for back mutation based on their possible influence on CDR conformation and/or binding to antigen. The placement of  
15 murine CDR regions with human variable framework region can result in conformational restraints, which, unless corrected by substitution of certain amino acid residues, lead to loss of binding affinity.

In one embodiment, the selection of amino acid residues for backmutation can be determined, in part, by computer modeling, using art recognized  
20 techniques. In general, molecular models are produced starting from solved structures for immunoglobulin chains or domains thereof. The chains to be modeled are compared for amino acid sequence similarity with chains or domains of solved three-dimensional structures, and the chains or domains showing the greatest sequence similarity is/are selected as starting points for construction of the molecular model. Chains or domains  
25 sharing at least 50% sequence identity are selected for modeling, and preferably those sharing at least 60%, 70%, 80%, 90% sequence identity or more are selected for modeling. The solved starting structures are modified to allow for differences between the actual amino acids in the immunoglobulin chains or domains being modeled, and those in the starting structure. The modified structures are then assembled into a  
30 composite immunoglobulin. Finally, the model is refined by energy minimization and by verifying that all atoms are within appropriate distances from one another and that bond lengths and angles are within chemically acceptable limits.

The selection of amino acid residues for substitution can also be determined, in part, by examination of the characteristics of the amino acids at particular  
35 locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids. For example, when an amino acid differs between a murine variable region framework residue and a selected human variable region framework residue, the human framework amino acid may be substituted by the equivalent

framework amino acid from the mouse binding molecule when it is reasonably expected that the amino acid: (1) noncovalently binds antigen directly, (2) is adjacent to a CDR region, (3) otherwise interacts with a CDR region (*e.g.*, is within about 3-6 Å of a CDR region as determined by computer modeling), or (4) participates in the VL-VH interface.

5               Residues which “noncovalently bind antigen directly” include amino acids in positions in framework regions which have a good probability of directly interacting with amino acids on the antigen according to established chemical forces, for example, by hydrogen bonding, Van der Waals forces, hydrophobic interactions, and the like.

10               Residues which are “adjacent to a CDR region” include amino acid residues in positions immediately adjacent to one or more of the CDRs in the primary sequence of the humanized immunoglobulin chain, for example, in positions immediately adjacent to a CDR as defined by Kabat, or a CDR as defined by Chothia (See *e.g.*, Chothia and Lesk *JMB* 196:901 (1987)). These amino acids are particularly  
15               likely to interact with the amino acids in the CDRs and, if chosen from the acceptor, may distort the donor CDRs and reduce affinity. Moreover, the adjacent amino acids may interact directly with the antigen (Amit *et al.*, *Science*, 233:747 (1986), which is incorporated herein by reference) and selecting these amino acids from the donor may be desirable to keep all the antigen contacts that provide affinity in the original binding  
20               molecule.

              Residues that “otherwise interact with a CDR region” include those that are determined by secondary structural analysis to be in a spatial orientation sufficient to effect a CDR region. In one embodiment, residues that “otherwise interact with a CDR region” are identified by analyzing a three-dimensional model of the donor  
25               immunoglobulin (*e.g.*, a computer-generated model). A three-dimensional model, typically of the original donor binding molecule, shows that certain amino acids outside of the CDRs are close to the CDRs and have a good probability of interacting with amino acids in the CDRs by hydrogen bonding, Van der Waals forces, hydrophobic interactions, *etc.* At those amino acid positions, the donor immunoglobulin amino acid  
30               rather than the acceptor immunoglobulin amino acid may be selected. Amino acids according to this criterion will generally have a side chain atom within about 3 Å of some atom in the CDRs and must contain an atom that could interact with the CDR atoms according to established chemical forces, such as those listed above.

              In the case of atoms that may form a hydrogen bond, the 3 Å is measured  
35               between their nuclei, but for atoms that do not form a bond, the 3 Å is measured between their Van der Waals surfaces. Hence, in the latter case, the nuclei must be within about 6 Å (3 Å plus the sum of the Van der Waals radii) for the atoms to be considered capable of interacting. In many cases the nuclei will be from 4 or 5 to 6 Å apart. In determining

whether an amino acid can interact with the CDRs, it is preferred not to consider the last 8 amino acids of heavy chain CDR as part of the CDRs, because from the viewpoint of structure, these 8 amino acids behave more as part of the framework.

Amino acids that are capable of interacting with amino acids in the CDRs, may be identified in yet another way. The solvent accessible surface area of each framework amino acid is calculated in two ways: (1) in the intact binding molecule, and (2) in a hypothetical molecule consisting of the binding molecule with its CDRs removed. A significant difference between these numbers of about 10 square angstroms or more shows that access of the framework amino acid to solvent is at least partly blocked by the CDRs, and therefore that the amino acid is making contact with the CDRs. Solvent accessible surface area of an amino acid may be calculated based on a three-dimensional model of an binding molecule, using algorithms known in the art (*e.g.*, Connolly, *J. Appl. Cryst.* 16:548 (1983) and Lee and Richards, *J. Mol. Biol.* 55:379 (1971), both of which are incorporated herein by reference). Framework amino acids may also occasionally interact with the CDRs indirectly, by affecting the conformation of another framework amino acid that in turn contacts the CDRs.

The amino acids at several positions in the framework are known to be capable of interacting with the CDRs in many binding molecules (Chothia and Lesk, *supra*, Chothia *et al.*, *supra* and Tramontano *et al.*, *J. Mol. Biol.* 215:175 (1990), all of which are incorporated herein by reference). Notably, the amino acids at positions 2, 48, 64 and 71 of the light chain and 26-30, 71 and 94 of the heavy chain (numbering according to Kabat) are known to be capable of interacting with the CDRs in many binding molecules. The amino acids at positions 35 in the light chain and 93 and 103 in the heavy chain are also likely to interact with the CDRs. At all these numbered positions, choice of the donor amino acid rather than the acceptor amino acid (when they differ) to be in the humanized immunoglobulin is preferred. On the other hand, certain residues capable of interacting with the CDR region, such as the first 5 amino acids of the light chain, may sometimes be chosen from the acceptor immunoglobulin without loss of affinity in the humanized binding molecule.

Residues which "participate in the VL-VH interface" or "packing residues" include those residues at the interface between VL and VH as defined, for example, by Novotny and Haber (*Proc. Natl. Acad. Sci. USA*, 82:4592-66 (1985)) or Chothia *et al.*, *supra*. Generally, unusual packing residues should be retained in the humanized binding molecule if they differ from those in the human frameworks.

In general, one or more of the amino acids fulfilling the above criteria is substituted. In some embodiments, all or most of the amino acids fulfilling the above criteria are substituted. Occasionally, there is some ambiguity about whether a particular amino acid meets the above criteria, and alternative variant binding molecules



are produced, one of which has that particular substitution, the other of which does not. Alternative variant binding molecules so produced can be tested in any of the assays described herein for the desired activity, and the preferred binding molecule selected.

Usually the CDR regions in humanized binding molecules are  
5 substantially identical, and more usually, identical to the corresponding CDR regions of the donor binding molecule. Although not usually desirable, it is sometimes possible to make one or more conservative amino acid substitutions of CDR residues without appreciably affecting the binding affinity of the resulting humanized binding molecule. By conservative substitutions it is meant combinations such as Gly, Ala; Val, Ile, Leu;  
10 Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

Additional candidates for substitution are acceptor human framework amino acids that are unusual or "rare" for a human immunoglobulin at that position. These amino acids can be substituted with amino acids from the equivalent position of the mouse donor binding molecule or from the equivalent positions of more typical  
15 human immunoglobulins. For example, substitution may be desirable when the amino acid in a human framework region of the acceptor immunoglobulin is rare for that position and the corresponding amino acid in the donor immunoglobulin is common for that position in human immunoglobulin sequences; or when the amino acid in the acceptor immunoglobulin is rare for that position and the corresponding amino acid in  
20 the donor immunoglobulin is also rare, relative to other human sequences. These criterion help ensure that an atypical amino acid in the human framework does not disrupt the binding molecule structure. Moreover, by replacing an unusual human acceptor amino acid with an amino acid from the donor binding molecule that happens to be typical for human binding molecules, the humanized binding molecule may be  
25 made less immunogenic.

The term "rare", as used herein, indicates an amino acid occurring at that position in less than about 20% but usually less than about 10% of sequences in a representative sample of sequences, and the term "common", as used herein, indicates an amino acid occurring in more than about 25% but usually more than about 50% of  
30 sequences in a representative sample. For example, all human light and heavy chain variable region sequences are respectively grouped into "subgroups" of sequences that are especially homologous to each other and have the same amino acids at certain critical positions (Kabat *et al.*, *supra*). When deciding whether an amino acid in a human acceptor sequence is "rare" or "common" among human sequences, it will often  
35 be preferable to consider only those human sequences in the same subgroup as the acceptor sequence.

Additional candidates for substitution are acceptor human framework amino acids that would be identified as part of a CDR region under the alternative



definition proposed by Chothia *et al.*, *supra*. Additional candidates for substitution are acceptor human framework amino acids that would be identified as part of a CDR region under the AbM and/or contact definitions. Notably, CDR1 in the variable heavy chain is defined as including residues 26-32.

5 Additional candidates for substitution are acceptor framework residues that correspond to a rare or unusual donor framework residue. Rare or unusual donor framework residues are those that are rare or unusual (as defined herein) for murine binding molecules at that position. For murine binding molecules, the subgroup can be determined according to Kabat and residue positions identified which differ from the  
10 consensus. These donor specific differences may point to somatic mutations in the murine sequence which enhances activity. Unusual residues that are predicted to affect binding are retained, whereas residues predicted to be unimportant for binding can be substituted.

Additional candidates for substitution are non-germline residues  
15 occurring in an acceptor framework region. For example, when an acceptor binding molecule chain (*i.e.*, a human binding molecule chain sharing significant sequence identity with the donor binding molecule chain) is aligned to a germline binding molecule chain (likewise sharing significant sequence identity with the donor chain), residues not matching between acceptor chain framework and the germline chain  
20 framework can be substituted with corresponding residues from the germline sequence.

Other than the specific amino acid substitutions discussed above, the framework regions of humanized binding molecules are usually substantially identical, and more usually, identical to the framework regions of the human binding molecules from which they were derived. Of course, many of the amino acids in the framework  
25 region make little or no direct contribution to the specificity or affinity of a binding molecule. Thus, many individual conservative substitutions of framework residues can be tolerated without appreciable change of the specificity or affinity of the resulting humanized binding molecule. Thus, in one embodiment the variable framework region of the humanized binding molecule shares at least 85% sequence identity to a human  
30 variable framework region sequence or consensus of such sequences. In another embodiment, the variable framework region of the humanized binding molecule shares at least 90%, preferably 95%, more preferably 96%, 97%, 98% or 99% sequence identity to a human variable framework region sequence or consensus of such sequences. In general, however, such substitutions are undesirable.

35 In one embodiment, a binding molecule of the invention further comprises at least one backmutation of a human amino acid residue to the corresponding mouse amino acid residue where the amino acid residue is an interface packing residue. "Interface packing residues" include those residues at the interface between VL and VH

as defined, for example, by Novotny and Haber, Proc. Natl. Acad. Sci. USA, 82:4592-66 (1985).

In one embodiment, a binding molecule of the invention further comprises at least one backmutation of a human amino acid residue to the corresponding mouse amino acid residue is a canonical residue. "Canonical residues" are conserved framework residues within a canonical or structural class known to be important for CDR conformation (Tramontano *et al.*, J. Mol. Biol. 215:175 (1990), all of which are incorporated herein by reference). Canonical residues include 2, 25, 27B, 28, 29, 30, 33, 48, 51, 52, 64, 71, 90, 94 and 95 of the light chain and residues 24, 26, 27 29, 34, 54, 55, 71 and 94 of the heavy chain. Additional residues (*e.g.*, CDR structure-determining residues) can be identified according to the methodology of Martin and Thorton (1996) J. Mol. Biol. 263:800.

In one embodiment, a binding molecule of the invention further comprises at least one backmutation of a human amino acid residue to the corresponding mouse amino acid residue where the amino acid residue is at a position capable of interacting with a CDR. Notably, the amino acids at positions 2, 48, 64 and 71 of the light chain and 26-30, 71 and 94 of the heavy chain (numbering according to Kabat) are known to be capable of interacting with the CDRs in many antibodies. The amino acids at positions 35 in the light chain and 93 and 103 in the heavy chain are also likely to interact with the CDRs.

Based on CLUSTAL W analysis, several amino acid residues in the human framework were identified for potential substitution, *e.g.*, with corresponding amino acid residues from the 6C8 light chain. These included positions 1, 8, 9, 10, 11, 13, 15, 17, 19, 20, 21, 22, 43, 45, 46, 58, 60, 63, 70, 76, 77, 78, 79, 83, 85, 87, 100, and 104.

In one embodiment, a variable light chain framework of a binding molecule of the invention further comprises at least one substitution of a human amino acid residue to the corresponding mouse amino acid residue selected from the group consisting of: E1D (*i.e.*, the E at position 1 of the CDR-grafted antibody which comprises murine CDRs and human FR regions is mutated to a D, which is the corresponding amino acid residue in the 6C8 antibody), P8Q, A9K, T10F, L11M, V13T, P15V, E17D, A19V, T20S, L21V, S22T, A43S, R45K, L46A, I58V, A60D, S63T, E70D, S76N, S77N, L78V, Q79H, F83L, V85E, Y87F, G100A, and V104L.

Based on CLUSTAL W analysis, several amino acid residues in the human framework were identified for potential substitution, *e.g.*, with corresponding amino acid residues from the 6C8 heavy chain. These included positions 5, 10, 11, 12, 15, 19, 23, 43, 46, 68, 77, 81, 83, 84, 86, 87, 89, 90, and 92.

In one embodiment, a variable heavy chain framework of a binding molecule of the invention further comprises at least one substitution of a human amino acid residue to the corresponding mouse amino acid residue selected from the group consisting of: R5K (i.e., the R at position 5 of the CDR-grafted antibody which  
5 comprises murine CDRs and human FR regions is mutated to a K, which is the corresponding amino acid residue in the 6C8 antibody), A10G, L11I, V12L, T15S, T19S, T23S, P43S, A46G, R68Q, K77R, V81F, T83K, M84I, N86S, M87V, P89T, V90A, and T92A.

The humanized binding molecules preferably exhibit a specific binding  
10 affinity for antigen of at least  $10^7$ ,  $10^8$ ,  $10^9$  or  $10^{10}$  M<sup>-1</sup>. Usually the upper limit of binding affinity of the humanized binding molecules for antigen is within a factor of three, four or five of that of the donor immunoglobulin. Often the lower limit of binding affinity is also within a factor of three, four or five of that of donor immunoglobulin. Alternatively, the binding affinity can be compared to that of a humanized binding  
15 molecule having no substitutions (e.g., a binding molecule having donor CDRs and acceptor FRs, but no FR substitutions). In such instances, the binding of the optimized binding molecule (with substitutions) is preferably at least two- to three-fold greater, or three- to four-fold greater, than that of the unsubstituted binding molecule. For making comparisons, activity of the various binding molecules can be determined, for example,  
20 by BIACORE (i.e., surface plasmon resonance using unlabelled reagents) or competitive binding assays.

Having conceptually selected the CDR and framework components of humanized binding molecules, a variety of methods are available for producing such binding molecules. Because of the degeneracy of the code, a variety of nucleic acid  
25 sequences will encode each binding molecule amino acid sequence. The desired nucleic acid sequences can be produced by *de novo* solid-phase DNA synthesis or by PCR mutagenesis of an earlier prepared variant of the desired polynucleotide.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion and insertion variants of target polypeptide DNA. See  
30 Adelman *et al.* (DNA 2:183 (1983)). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a single-stranded DNA template. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that incorporates the oligonucleotide primer, and encodes the selected alteration in the target polypeptide DNA.

35 The variable segments of binding molecules produced as described *supra* (e.g., the heavy and light chain variable regions of chimeric, humanized, or human binding molecules) are typically linked to at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Human constant region

DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B cells (see Kabat *et al.*, *supra*, and Liu *et al.*, W087/02671) (each of which is incorporated by reference in its entirety for all purposes). Ordinarily, the binding molecule will contain both light chain and heavy chain constant regions. The heavy chain constant region usually includes CH1, hinge, CH2, CH3, and CH4 regions. A binding molecule described herein include antibodies having all types of constant regions, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. The choice of constant region depends, in part, or whether binding molecule-dependent complement and/or cellular mediated toxicity is desired. For example, isotopes IgG1 and IgG3 have complement activity and isotopes IgG2 and IgG4 do not. When it is desired that the binding molecule (*e.g.*, humanized binding molecule) exhibit cytotoxic activity, the constant domain is usually a complement fixing constant domain and the class is typically IgG1. When such cytotoxic activity is not desirable, the constant domain may be, *e.g.*, of the IgG2 class. Choice of isotype can also affect passage of antibody into the brain. Human isotype IgG1 is preferred. Light chain constant regions can be lambda or kappa. The humanized binding molecule may comprise sequences from more than one class or isotype. Binding molecules can be expressed as tetramers containing two light and two heavy chains, as separate heavy chains, light chains, as Fab, Fab' F(ab')<sub>2</sub>, and Fv, or as single chain binding molecules in which heavy and light chain variable domains are linked through a spacer.

### III. Production of Binding Molecules

The present invention features binding molecules having specificity for GTR, *e.g.*, human GTR. Such binding molecules can be used in formulating various therapeutic compositions of the invention or, preferably, provide complementarity determining regions for the production of humanized or chimeric binding molecules (described in detail below). The production of non-human monoclonal binding molecules, *e.g.*, murine, guinea pig, primate, rabbit or rat, can be accomplished by, for example, immunizing the animal with GTR or with a nucleic acid molecule encoding GTR. For example, the 6C8 binding molecule was made by placing the gene encoding human GTR in an expression vector and immunizing animals. A longer polypeptide comprising GTR or an immunogenic fragment of GTR or anti-idiotypic binding molecule of GTR can also be used. (see, for example, Harlow & Lane, *supra*, incorporated by reference for all purposes). Such an immunogen can be obtained from a natural source, by peptide synthesis or by recombinant expression. Optionally, the immunogen can be administered, fused or otherwise complexed with a carrier protein, as



described below. Optionally, the immunogen can be administered with an adjuvant. The term "adjuvant" refers to a compound that when administered in conjunction with an antigen augments the immune response to the antigen, but when administered alone does not generate an immune response to the antigen. Adjuvants can augment an  
5 immune response by several mechanisms including lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages. Several types of adjuvants can be used as described below. Complete Freund's adjuvant followed by incomplete adjuvant is preferred for immunization of laboratory animals.

Rabbits or guinea pigs are typically used for making polyclonal binding  
10 molecules. Exemplary preparation of polyclonal binding molecules, *e.g.*, for passive protection, can be performed as follows. Animals are immunized with 100  $\mu$ g GTR, plus adjuvant, and euthanized at 4-5 months. Blood is collected and IgG is separated from other blood components. Binding molecules specific for the immunogen may be partially purified by affinity chromatography. An average of about 0.5-1.0 mg of  
15 immunogen-specific binding molecule is obtained per animal, giving a total of 60-120 mg.

Mice are typically used for making monoclonal binding molecules. Monoclonals can be prepared against a fragment by injecting the fragment or longer form of GTR into a mouse, preparing hybridomas and screening the hybridomas for a  
20 binding molecule that specifically binds to GTR. Optionally, binding molecules are screened for binding to a specific region or desired fragment of GTR without binding to other nonoverlapping fragments of GTR. The latter screening can be accomplished by determining binding of a binding molecule to a collection of deletion mutants of a GTR peptide and determining which deletion mutants bind to the binding molecule. Binding  
25 can be assessed, for example, by Western blot or ELISA. The smallest fragment to show specific binding to the binding molecule defines the epitope of the binding molecule. Alternatively, epitope specificity can be determined by a competition assay in which a test and reference binding molecule compete for binding to GTR. If the test and reference binding molecule compete, then they bind to the same epitope (or epitopes  
30 sufficiently proximal) such that binding of one binding molecule interferes with binding of the other. The preferred isotype for such binding molecules is mouse isotype IgG2a or equivalent isotype in other species. Mouse isotype IgG2a is the equivalent of human isotype IgG1.

In another embodiment, DNA encoding a binding molecule may be readily  
35 isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine binding molecules). The isolated and subcloned hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression

vectors, which are then transfected into prokaryotic or eukaryotic host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells or myeloma cells that do not otherwise produce immunoglobulins. More particularly, the isolated DNA (which may be synthetic as described herein) may be used to clone constant and variable region sequences  
5 for the manufacture of binding molecules as described in Newman *et al.*, U.S. Pat. No. 5,658,570, filed January 25, 1995, which is incorporated by reference herein. Essentially, this entails extraction of RNA from the selected cells, conversion to cDNA, and amplification by PCR using Ig specific primers. Suitable primers for this purpose are also described in U.S. Pat. No. 5,658,570. Transformed cells expressing the desired antibody  
10 may be produced in relatively large quantities to provide clinical and commercial supplies of the binding molecule.

Those skilled in the art will also appreciate that DNA encoding binding molecules or fragments thereof (e.g., antigen binding sites) may also be derived from antibody phage libraries, e.g., using pd phage or Fd phagemid technology. Exemplary  
15 methods are set forth, for example, in EP 368 684 B1; U.S. patent. 5,969,108, Hoogenboom, H.R. and Chames. 2000. *Immunol. Today* 21:371; Nagy et al. 2002. *Nat. Med.* 8:801; Huie et al. 2001. *Proc. Natl. Acad. Sci. USA* 98:2682; Lui et al. 2002. *J. Mol. Biol.* 315:1063, each of which is incorporated herein by reference. Several publications (e.g., Marks et al. *Bio/Technology* 10:779-783 (1992)) have described the production of  
20 high affinity human binding molecules by chain shuffling, as well as combinatorial infection and *in vivo* recombination as a strategy for constructing large phage libraries. In another embodiment, Ribosomal display can be used to replace bacteriophage as the display platform (see, e.g., Hanes et al. 2000. *Nat. Biotechnol.* 18:1287; Wilson et al. 2001. *Proc. Natl. Acad. Sci. USA* 98:3750; or Irving et al. 2001 *J. Immunol. Methods*  
25 248:31. In yet another embodiment, cell surface libraries can be screened for binding molecules (Boder et al. 2000. *Proc. Natl. Acad. Sci. USA* 97:10701; Daugherty et al. 2000 *J. Immunol. Methods* 243:211. Such procedures provide alternatives to traditional hybridoma techniques for the isolation and subsequent cloning of monoclonal binding molecules.

30 Yet other embodiments of the present invention comprise the generation of human or substantially human binding molecules in transgenic animals (e.g., mice) that are incapable of endogenous immunoglobulin production (see e.g., U.S. Pat. Nos. 6,075,181, 5,939,598, 5,591,669 and 5,589,369 each of which is incorporated herein by reference). For example, it has been described that the homozygous deletion of the  
35 antibody heavy-chain joining region in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of a human immunoglobulin gene array to such germ line mutant mice will result in the production of human binding molecules upon antigen challenge. Another preferred means of

generating human binding molecules using SCID mice is disclosed in U.S. Pat. No. 5,811,524 which is incorporated herein by reference. It will be appreciated that the genetic material associated with these human binding molecules may also be isolated and manipulated as described herein.

5 Yet another highly efficient means for generating recombinant binding molecules is disclosed by Newman, *Biotechnology*, 10: 1455-1460 (1992). Specifically, this technique results in the generation of primatized binding molecules that contain monkey variable domains and human constant sequences. This reference is incorporated by reference in its entirety herein. Moreover, this technique is also described in U.S.  
10 Pat. Nos. 5,658,570, 5,693,780 and 5,756,096 each of which is incorporated herein by reference.

In another embodiment, lymphocytes can be selected by micromanipulation and the variable genes isolated. For example, peripheral blood mononuclear cells can be isolated from an immunized mammal and cultured for about 7  
15 days in vitro. The cultures can be screened for specific IgGs that meet the screening criteria. Cells from positive wells can be isolated. Individual Ig-producing B cells can be isolated by FACS or by identifying them in a complement-mediated hemolytic plaque assay. Ig-producing B cells can be micromanipulated into a tube and the VH and VL genes can be amplified using, e.g., RT-PCR. The VH and VL genes can be cloned into  
20 an antibody expression vector and transfected into cells (e.g., eukaryotic or prokaryotic cells) for expression.

Moreover, genetic sequences useful for producing the polypeptides of the present invention may be obtained from a number of different sources. For example, as discussed extensively above, a variety of human antibody genes are available in the form  
25 of publicly accessible deposits. Many sequences of antibodies and antibody-encoding genes have been published and suitable antibody genes can be chemically synthesized from these sequences using art recognized techniques. Oligonucleotide synthesis techniques compatible with this aspect of the invention are well known to the skilled artisan and may be carried out using any of several commercially available automated  
30 synthesizers. In addition, DNA sequences encoding several types of heavy and light chains set forth herein can be obtained through the services of commercial DNA synthesis vendors. The genetic material obtained using any of the foregoing methods may then be altered or synthetic to provide obtain polypeptides of the present invention.

Alternatively, antibody-producing cell lines may be selected and cultured  
35 using techniques well known to the skilled artisan. Such techniques are described in a variety of laboratory manuals and primary publications. In this respect, techniques suitable for use in the invention as described below are described in *Current Protocols in Immunology*, Coligan et al., Eds., Green Publishing Associates and Wiley-Interscience,



John Wiley and Sons, New York (1991) which is herein incorporated by reference in its entirety, including supplements.

As is well known, RNA may be isolated from the original hybridoma cells or from other transformed cells by standard techniques, such as guanidinium  
5 isothiocyanate extraction and precipitation followed by centrifugation or chromatography. Where desirable, mRNA may be isolated from total RNA by standard techniques such as chromatography on oligo dT cellulose. Suitable techniques are familiar in the art.

In one embodiment, cDNAs that encode the light and the heavy chains of  
10 the binding molecule may be made, either simultaneously or separately, using reverse transcriptase and DNA polymerase in accordance with well known methods. PCR may be initiated by consensus constant region primers or by more specific primers based on the published heavy and light chain DNA and amino acid sequences. As discussed above, PCR also may be used to isolate DNA clones encoding the binding molecule light  
15 and heavy chains. In this case the libraries may be screened by consensus primers or larger homologous probes, such as mouse constant region probes.

DNA, typically plasmid DNA, may be isolated from the cells using techniques known in the art, restriction mapped and sequenced in accordance with standard, well known techniques set forth in detail, e.g., in the foregoing references  
20 relating to recombinant DNA techniques. Of course, the DNA may be synthetic according to the present invention at any point during the isolation process or subsequent analysis.

In one embodiment, a binding molecule of the invention comprises or consists of an antigen binding fragment of an antibody. The term “antigen-binding  
25 fragment” refers to a polypeptide fragment of an immunoglobulin or antibody that binds antigen or competes with intact antibody (*i.e.*, with the intact antibody from which they were derived) for antigen binding (*i.e.*, specific binding). As used herein, the term “fragment” of an antibody molecule includes antigen-binding fragments of antibodies, for example, an antibody light chain (VL), an antibody heavy chain (VH), a single chain  
30 antibody (scFv), a F(ab')<sub>2</sub> fragment, a Fab fragment, an Fd fragment, an Fv fragment, and a single domain antibody fragment (DAb). Fragments can be obtained, e.g., *via* chemical or enzymatic treatment of an intact or complete antibody or antibody chain or by recombinant means.

In one embodiment, a binding molecule of the invention is an engineered  
35 or modified antibody. Engineered forms of antibodies include, for example, minibodies,



diabodies, diabodies fused to CH3 molecules, tetravalent antibodies, intradiabodies (e.g., Jendreyko et al. 2003. *J. Biol. Chem.* 278:47813), bispecific antibodies, fusion proteins (e.g., antibody cytokine fusion proteins) or, bispecific antibodies. Other immunoglobulins (Ig) and certain variants thereof are described, for example in U.S. Pat. No. 4,745,055; EP 256,654; Faulkner et al., *Nature* 298:286 (1982); EP 120,694; EP 125,023; Morrison, *J. Immun.* 123:793 (1979); Kohler et al., *Proc. Natl. Acad. Sci. USA* 77:2197 (1980); Raso et al., *Cancer Res.* 41:2073 (1981); Morrison et al., *Ann. Rev. Immunol.* 2:239 (1984); Morrison, *Science* 229:1202 (1985); Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559.

Reassorted immunoglobulin chains also are known. See, for example, U.S. Pat. No. 4,444,878; WO 88/03565; and EP 68,763 and references cited therein.

In one embodiment, the modified antibodies of the invention are minibodies. Minibodies are dimeric molecules made up of two polypeptide chains each comprising an ScFv molecule (a single polypeptide comprising one or more antigen binding sites, e.g., a VL domain linked by a flexible linker to a VH domain fused to a CH3 domain via a connecting peptide).

ScFv molecules can be constructed in a VH-linker-VL orientation or VL-linker-VH orientation.

The flexible hinge that links the VL and VH domains that make up the antigen binding site preferably comprises from about 10 to about 50 amino acid residues. An exemplary connecting peptide for this purpose is (Gly4Ser)3 (SEQ ID NO:17) (Huston et al. 1988. *Proc. Natl. Acad. Sci. USA* 85:5879). Other connecting peptides are known in the art.

Methods of making single chain antibodies are well known in the art, e.g., Ho et al. 1989. *Gene* 77:51; Bird et al. 1988 *Science* 242:423; Pantoliano et al. 1991. *Biochemistry* 30:10117; Milenic et al. 1991. *Cancer Research* 51:6363; Takkinen et al. 1991. *Protein Engineering* 4:837.

Minibodies can be made by constructing an ScFv component and connecting peptide-CH3 component using methods described in the art (see, e.g., US patent 5,837,821 or WO 94/09817A1). These components can be isolated from separate plasmids as restriction fragments and then ligated and recloned into an appropriate vector. Appropriate assembly can be verified by restriction digestion and DNA sequence analysis.

Diabodies are similar to scFv molecules, but usually have a short (less than 10 and preferably 1-5) amino acid residue linker connecting both V-domains, such that the VL and VH domains on the same polypeptide chain can not interact. Instead, the VL and VH domain of one polypeptide chain interact with the VH and VL domain (respectively) on a second polypeptide chain (WO 02/02781). In one embodiment, a binding molecule of the invention is a diabody fused to at least one heavy chain portion. In a preferred embodiment, a binding molecule of the invention is a diabody fused to a CH3 domain.

Other forms of modified antibodies are also within the scope of the instant invention (e.g., WO 02/02781 A1; 5,959,083; 6,476,198 B1; US 2002/0103345 A1; WO 00/06605; Byrn et al. 1990. Nature. 344:667-70; Chamow and Ashkenazi. 1996. Trends Biotechnol. 14:52).

In one embodiment, a binding molecule of the invention comprises an immunoglobulin constant region. It is known in the art that the constant region mediates several effector functions. For example, binding of the C1 component of complement to binding molecules activates the complement system. Activation of complement is important in the opsonisation and lysis of cell pathogens. The activation of complement also stimulates the inflammatory response and may also be involved in autoimmune hypersensitivity. Further, binding molecules bind to cells via the Fc region, with a Fc receptor site on the binding molecule Fc region binding to a Fc receptor (FcR) on a cell. There are a number of Fc receptors which are specific for different classes of binding molecule, including IgG (gamma receptors), IgE (epsilon receptors), IgA (alpha receptors) and IgM (mu receptors). Binding of binding molecule to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction of binding molecule-coated particles, clearance of immune complexes, lysis of binding molecule-coated target cells by killer cells (called antibody-dependent cell-mediated cytotoxicity, or ADCC), release of inflammatory mediators, placental transfer and control of immunoglobulin production.

In one embodiment, effector functions may be eliminated or reduced by using a constant region of an IgG4 binding molecule, which is thought to be unable to deplete target cells, or making Fc variants, wherein residues in the Fc region critical for effector function(s) are mutated using techniques known in the art, for example, U.S. Pat. No. 5,585,097. For example, the deletion or inactivation (through point mutations

or other means) of a constant region domain may reduce Fc receptor binding of the circulating modified binding molecule thereby increasing tumor localization. In other cases it may be that constant region modifications consistent with the instant invention moderate complement binding and thus reduce the serum half life and nonspecific  
5 association of a conjugated cytotoxin. Yet other modifications of the constant region may be used to modify disulfide linkages or oligosaccharide moieties that allow for enhanced localization due to increased antigen specificity or binding molecule flexibility. More generally, those skilled in the art will realize that binding molecules modified as described herein may exert a number of subtle effects that may or may not  
10 be readily appreciated. However the resulting physiological profile, bioavailability and other biochemical effects of the modifications, such as tumor localization, biodistribution and serum half-life, may easily be measured and quantified using well known immunological techniques without undue experimentation.

In one embodiment, a binding molecule of the invention can be  
15 derivatized or linked to another functional molecule (*e.g.*, another peptide or protein). Accordingly, a binding molecule of the invention include derivatized and otherwise modified forms of the anti-GITR binding molecules described herein, including immunoadhesion molecules. For example, a binding molecule of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or  
20 otherwise) to one or more other molecular entities, such as another binding molecule (*e.g.*, a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate association of the binding molecule with another molecule (such as a streptavidin core region or a polyhistidine tag).

25 One type of derivatized binding molecule is produced by crosslinking two or more binding molecules (of the same type or of different types, *e.g.*, to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) or homobifunctional (*e.g.*,  
30 disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

Useful detectable agents with which a binding molecule of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin and the like. A binding molecule may also  
35 be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish

peroxidase, glucose oxidase and the like. When a binding molecule is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and  
5 diaminobenzidine leads to a colored reaction product, which is detectable. A binding molecule may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

#### 10 IV. *Expression of Binding molecules*

A binding molecule of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express a binding molecule recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and  
15 heavy chains of the binding molecule such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium a binding molecule can be recovered. Standard recombinant DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors, and introduce the  
20 vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss, *et al.*

To express a binding molecule of the invention, DNAs encoding partial  
25 or full-length light and heavy chains may be inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" means that a binding molecule gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the  
30 binding molecule gene. In one embodiment, the expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The binding molecule light chain gene and the binding molecule heavy chain gene may be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The binding molecule genes may be inserted into the expression  
35 vector by standard methods (*e.g.*, ligation of complementary restriction sites on the binding molecule gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the binding molecule light or heavy chain sequences, the expression vector may already carry binding molecule constant region sequences.



For example, one approach to converting VH and VL sequences to full-length binding molecule genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is  
5 operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the binding molecule chain from a host cell. The binding molecule chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the binding molecule chain gene. The signal peptide can be an  
10 immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

In addition to the binding molecule chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the binding molecule chain genes in a host cell. The term "regulatory  
15 sequence" includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the binding molecule chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It will be appreciated by those skilled in the art that the design  
20 of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the  
25 CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner, *et al.*

30 In addition to the binding molecule chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents  
35 Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in

dhfr<sup>-</sup> host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the binding molecule heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. It is possible to express a binding molecule of the invention in either prokaryotic or eukaryotic host cells, expression of binding molecules in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active binding molecule.

Commonly, expression vectors contain selection markers (*e.g.*, ampicillin-resistance, hygromycin-resistance, tetracycline resistance or neomycin resistance) to permit detection of those cells transformed with the desired DNA sequences (see, *e.g.*, Itakura *et al.*, US Patent 4,704,362).

*E. coli* is one prokaryotic host particularly useful for cloning the polynucleotides (*e.g.*, DNA sequences) of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (*e.g.*, an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (*trp*) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, are also useful for expression. *Saccharomyces* is a preferred yeast host, with suitable vectors having expression control sequences (*e.g.*, promoters), an origin of replication, termination sequences and the like as desired. Typical promoters include 3-phosphoglycerate kinase and other glycolytic enzymes. Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase, isocytochrome C, and enzymes responsible for maltose and galactose utilization.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (*e.g.*, polynucleotides encoding binding molecules). See Winnacker, *From Genes to Clones*,

VCH Publishers, N.Y., N.Y. (1987). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting heterologous proteins (*e.g.*, intact binding molecules) have been developed in the art, and include CHO cell lines, various Cos cell lines, HeLa cells, myeloma cell lines, or transformed B-cells or hybridomas.

5 Preferably, the cells are nonhuman. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer (Queen *et al.*, *Immunol. Rev.* 89:49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences

10 are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papilloma virus, cytomegalovirus and the like. See Co *et al.*, *J. Immunol.* 148:1149 (1992).

Alternatively, binding molecule-coding sequences can be incorporated in transgenes for introduction into the genome of a transgenic animal and subsequent expression in the milk of the transgenic animal (see, *e.g.*, Deboer *et al.*, US 5,741,957, Rosen, US 5,304,489, and Meade *et al.*, US 5,849,992). Suitable transgenes include

15 coding sequences for light and/or heavy chains in operable linkage with a promoter and enhancer from a mammary gland specific gene, such as casein or beta lactoglobulin.

Preferred mammalian host cells for expressing the recombinant binding molecules of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells.

20 When recombinant expression vectors encoding binding molecule genes are introduced into mammalian host cells, binding molecules are produced by culturing the host cells for a period of time sufficient to allow for expression of the binding molecule in the host cells or, more preferably, secretion of the binding molecule into the culture medium in which the host cells are grown. Binding molecules can be recovered from the culture medium using standard protein purification methods.

25

The vectors containing the polynucleotide sequences of interest (*e.g.*, the binding molecule heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection may be used for other

30 cellular hosts. (See generally Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, 2nd ed., 1989) (incorporated by reference in its entirety for all purposes). Other methods used to transform mammalian cells include the use of polybrene, protoplast fusion, liposomes, electroporation, and microinjection (see

35



generally, Sambrook *et al.*, *supra*). For production of transgenic animals, transgenes can be microinjected into fertilized oocytes, or can be incorporated into the genome of embryonic stem cells, and the nuclei of such cells transferred into enucleated oocytes.

When heavy and light chains are cloned on separate expression vectors,  
5 the vectors are co-transfected to obtain expression and assembly of intact immunoglobulins. Once expressed, the whole binding molecules, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, HPLC  
10 purification, gel electrophoresis and the like (see generally Scopes, Protein Purification (Springer-Verlag, N.Y., (1982))). Substantially pure binding molecules of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses.

Host cells can also be used to produce portions of intact binding  
15 molecules, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of a binding molecule of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA  
20 encoding either or both of the light and heavy chains that is not necessary for binding to GITR. The molecules expressed from such truncated DNA molecules are also encompassed by a binding molecule of the invention. In addition, bifunctional binding molecules may be produced in which one heavy and one light chain are a binding molecule of the invention and the other heavy and light chain are specific for an antigen  
25 other than GITR by crosslinking a binding molecule of the invention to a second binding molecule by standard chemical crosslinking methods.

In view of the foregoing, another aspect of the invention pertains to nucleic acid, vector and host cell compositions that can be used for recombinant expression of a binding molecule of the invention. The nucleotide sequence encoding  
30 the 6C8 light chain variable region is shown in Figure 18 and SEQ ID NO.: 10. The CDR1 domain of the VL encompasses nucleotides 130-162 of SEQ ID NO:10 (SEQ ID NO:14), the CDR2 domain encompasses nucleotides 208-228 of SEQ ID NO:10 (SEQ ID NO:15) and the CDR3 domain encompasses nucleotides 325-351 of SEQ ID NO:10 (SEQ ID NO:16). The nucleotide sequence encoding the 6C8 heavy chain variable  
35 region is also shown in Figure 18 and SEQ ID NO.: 9. The CDR1 domain of the VH encompasses nucleotides 133-168 of SEQ ID NO:9 (SEQ ID NO:11), the CDR2 domain encompasses nucleotides 211-258 of SEQ ID NO:9 (SEQ ID NO:12) and the CDR3 domain encompasses nucleotides 355-381 of SEQ ID NO:9 (SEQ ID NO:13). In one



embodiment, the nucleotide sequence encoding CDR2 of the VH comprises SEQ ID NO:12. In another embodiment, the nucleotide sequence encoding CDR2 of the VH comprises SEQ ID NO:65

(CACATTTGGTGGGATGATGATAAGTACTATCAACCATCCCTGAAGAGC). It  
5 will be appreciated by the skilled artisan that nucleotide sequences encoding 6C8-related binding molecules can be derived from the nucleotide sequences encoding the 6C8 VL and VH using the genetic code and standard molecular biology techniques.

In one embodiment, the invention provides isolated nucleic acid molecules encoding a polypeptide sequence comprising a 6C8 CDR, *e.g.*, comprising an  
10 amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:19, SEQ ID NO:5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8.

In still another embodiment, the invention provides an isolated nucleic acid molecule encoding a binding molecule light chain variable region comprising the amino acid sequence of SEQ ID NO: 2, although the skilled artisan will appreciate that  
15 due to the degeneracy of the genetic code, other nucleic acid molecules can encode the amino acid sequence of SEQ ID NO: 2. The nucleic acid molecule can encode only the VL or can also encode a binding molecule light chain constant region, operatively linked to the VL. In one embodiment, this nucleic acid molecule is in a recombinant expression vector.

In still another embodiment, the invention provides an isolated nucleic acid molecule encoding a binding molecule heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1, although the skilled artisan will appreciate that due to the degeneracy of the genetic code, other nucleic acid molecules can encode the amino acid sequence of SEQ ID NO: 1. In another embodiment, the invention provides  
25 an isolated nucleic acid molecule encoding a binding molecule heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 66, although the skilled artisan will appreciate that due to the degeneracy of the genetic code, other nucleic acid molecules can encode the amino acid sequence of SEQ ID NO: 66. The nucleic acid molecule can encode only the VH or can also encode a heavy chain constant region,  
30 operatively linked to the VH. For example, the nucleic acid molecule can comprise an IgG1 or IgG2 constant region. In one embodiment, this nucleic acid molecule is in a recombinant expression vector.

The invention also provides recombinant expression vectors encoding a binding molecule heavy chain and/or a binding molecule light chain. For example, in  
35 one embodiment, the invention provides a recombinant expression vector encoding:

a) a binding molecule light chain having a variable region comprising the amino acid sequence of SEQ ID NO: 2; and

b) a binding molecule heavy chain having a variable region comprising the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the invention provides a recombinant expression vector encoding:

5 a) a binding molecule light chain having a variable region comprising the amino acid sequence of SEQ ID NO: 2; and

b) a binding molecule heavy chain having a variable region comprising the amino acid sequence of SEQ ID NO: 66.

10 The invention also provides host cells into which one or more of the recombinant expression vectors of the invention have been introduced. Preferably, the host cell is a mammalian host cell.

Still further the invention provides a method of synthesizing a recombinant binding molecules of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant binding molecule of the invention is  
15 synthesized. The method may further comprise isolating the recombinant binding molecule from the culture medium.

#### ***V. Uses of Binding Molecules of the Invention***

Given their ability to bind to GITR, the binding molecules of the  
20 invention may be used to detect GITR (*e.g.*, in a biological sample, such as serum or plasma), using a conventional immunoassay, such as an enzyme linked immunosorbent assays (ELISA), a radioimmunoassay (RIA) or tissue immunohistochemistry. The invention provides a method for detecting hGITR in a biological sample comprising contacting a biological sample with a binding molecule of the invention and detecting  
25 either the binding molecule bound to hGITR or unbound binding molecule, to thereby detect hGITR in the biological sample. The method may be performed *in vitro* or *in vivo*. The binding molecule is directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound binding molecule. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials,  
30 luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein,  
35 dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ .

Alternative to labeling the binding molecule, hGITR can be assayed in biological fluids by a competition immunoassay utilizing GITR standards labeled with a

detectable substance and an unlabeled anti-hGITR binding molecule. In this assay, the biological sample, the labeled GITR standards and the anti-hGITR binding molecule are combined and the amount of labeled GITR standard bound to the unlabeled binding molecule is determined. The amount of hGITR in the biological sample is inversely  
5 proportional to the amount of labeled GITR standard bound to the anti-hGITR binding molecule.

An anti-GITR binding molecule of the invention can also be used to detect GITRs in samples from species other than humans, in particular GITRs from primates (*e.g.*, chimpanzee, baboon, marmoset, cynomolgus and rhesus).

10 In another embodiment, the invention provides a method for abrogating the suppression of T effector cells by T regulatory cells. Abrogation of suppression of T effector cells by T regulatory cells can be assayed, for example, by measuring the ability of the binding molecule to enhance T cell effector function in the presence of T regulatory cells, *e.g.*, cytokine production, (*e.g.*, IL-2 production) or cell proliferation  
15 (*e.g.*, T helper cell proliferation), by, for example, measuring <sup>3</sup>H-thymidine incorporation or by FACS analysis. For example, the response or activity of T effector cells will be low in the presence of T regulatory cells, but will increase with the addition of a GITR binding molecule even if T regulatory cells are present, *i.e.*, a GITR binding molecules abrogates the suppression of T effector cells by T regulatory cells.

20 The binding molecules of the invention may also be used to attenuate the degradation of I- $\kappa$ B in cells. Attenuated degradation of I- $\kappa$ B in cells can be assayed, for example, by Western blotting and quantitating the amount of I- $\kappa$ B following treatment of cells with anti-GITR binding molecule.

Numerous disease or pathological conditions would benefit from  
25 enhancing the activity of T effector cells and/or downmodulating the activity of T regulatory cells, *e.g.*, by abrogating the suppression of T effector cells by T regulatory cells. For example, immune effector cells often fail to react effectively with cancer cells. Accordingly, when an enhanced effector T cell or antibody response is desired, the methods of the invention can be used to treat a subject suffering from such a disorder.  
30 In one embodiment such methods comprise administering to the subject a binding molecule of the invention such that suppression of T effector cells by T regulatory cells is abrogated, thereby enhancing an immune response. Preferably, the subject is a human subject. Alternatively, the subject can be a mammal expressing a GITR with which a binding molecule of the invention cross-reacts. Still further, the subject can be a  
35 mammal into which GITR has been introduced (*e.g.*, by administration of GITR or by expression of a GITR transgene). A binding molecule of the invention may be administered to a human subject for therapeutic or prophylactic purposes. For example, the subject may have been diagnosed as having the disease or disorder or may be

predisposed or susceptible to the disease. Moreover, a binding molecule of the invention can be administered to a non-human mammal expressing a GITR molecule with which the binding molecule cross-reacts (*e.g.*, a primate) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be  
5 useful for evaluating the therapeutic and/or prophylactic efficacy of binding molecules of the invention (*e.g.*, testing of dosages and/or time courses of administration).

Exemplary uses of the binding molecules of the invention are discussed further below:

#### 10 *Immunostimulatory Compositions*

As described in the appended examples, the binding molecules of the invention can be used as immunostimulatory compositions (or vaccines), *e.g.*, in combination with an antigen, to promote an enhanced immune response to an antigen of interest, *e.g.*, a protein antigen, in a subject. That is, the binding molecules of the invention can serve as  
15 adjuvants to enhance immune responses. For example, to stimulate an antibody or cellular immune response to an antigen of interest (*e.g.*, for vaccination purposes), the antigen and a binding molecules of the invention can be coadministered (*e.g.*, coadministered at the same time in the same or separate compositions, or sequentially in time) such that an enhanced immune response occurs. The antigen of interest and a  
20 binding molecule can be formulated together into a single pharmaceutical composition or in separate compositions. In one embodiment, the antigen of interest and the binding molecule are administered simultaneously to the subject. Alternatively, in certain situations it may be desirable to administer the antigen first and then the binding molecule or *vice versa* (for example, it may be beneficial to first administer the antigen alone to stimulate a response and then administer a binding molecule, alone or together  
25 with a boost of antigen). In preferred embodiments, a GITR binding molecule of the invention is administered at the time of priming with antigen, *i.e.*, at the time of the first administration of antigen. For example, day -3, -2, -1, 0, +1, +2, +3. A particularly preferred day of administration of a GITR binding molecule of the invention is day -1  
30 prior to administration of antigen.

An antigen of interest is, for example, one capable of providing protection in subject against challenge by an infectious agent from which the antigen was derived, or which is capable of affecting tumor growth and metastasis in a manner which is of benefit to a subject. Exemplary antigens of interest therefore include those  
35 derived from infectious agents, cancer cells, and the like, wherein an immune response directed against the antigen serves to prevent or treat disease caused by the agent. Such antigens include, but are not limited to, viral, bacterial, fungal or parasite proteins, glycoproteins, lipoproteins, glycolipids, and the like. Antigens of interest also include



those which provide benefit to a subject which is at risk for acquiring or which is diagnosed as having a tumor and may include, *e.g.*, tumor-related antigens expressed exclusively by or at increased levels by tumor cells. The subject is preferably a mammal and most preferably, is a human.

5                   As used herein the term "pathogen" or "pathogenic agent" includes microorganisms that are capable of infecting or parasitizing normal hosts (*e.g.*, animals (such as mammals, preferably primates, *e.g.* humans)). As used herein, the term also includes opportunistic agents, *e.g.*, microorganisms that are capable of infecting or parasitizing abnormal hosts, *e.g.*, hosts in which normal flora have been supplanted, *e.g.*,  
10 as a result of a treatment regimen, or immunocompromised hosts. As used herein the term also includes microorganisms whose replication is unwanted in a subject or toxic molecules (*e.g.*, toxins) produced by microorganisms.

Non-limiting examples of viral antigens include, but are not limited to, the nucleoprotein (NP) of influenza virus and the Gag proteins of HIV. Other  
15 heterologous antigens include, but are not limited to, HIV Env protein or its component parts, gp120 and gp41, HIV Nef protein, and the HIV Pol proteins, reverse transcriptase and protease. In addition, other viral antigens such as Ebola virus (EBOV) antigens, such as, for example, EBOV NP or glycoprotein (GP), either full-length or GP deleted in the mucin region of the molecule (Yang Z-Y, *et al.* (2000) *Nat Med* 6:886-9, 2000),  
20 small pox antigens, hepatitis A, B or C virus, human rhinovirus such as type 2 or type 14, Herpes simplex virus, poliovirus type 2 or 3, foot-and-mouth disease virus (FMDV), rabies virus, rotavirus, influenza virus, coxsackie virus, human papilloma virus (HPV), for example the type 16 papilloma virus, the E7 protein thereof, and fragments containing the E7 protein or its epitopes; and simian immunodeficiency virus (SIV) may  
25 be used. The antigens of interest need not be limited to antigens of viral origin. Parasitic antigens, such as, for example, malarial antigens are included, as are fungal antigens, bacterial antigens and tumor antigens can also be used in connection with the disclosed compositions and methods. Non-limiting examples of bacterial antigens include: *Bordetella pertussis* (*e.g.*, P69 protein and filamentous haemagglutinin (FHA) antigens), *Vibrio cholerae*, *Bacillus anthracis*, and *E. coli* antigens such as *E. coli* heat  
30 Labile toxin B subunit (LT-B), *E. coli* K88 antigens, and enterotoxigenic *E. coli* antigens. Other examples of antigens include *Schistosoma mansoni* P28 glutathione S-transferase antigens (P28 antigens) and antigens of flukes, mycoplasma, roundworms, tapeworms, *Chlamydia trachomatis*, and malaria parasites, *e.g.*, parasites of the genus  
35 plasmodium or babesia, for example *Plasmodium falciparum*, and peptides encoding immunogenic epitopes from the aforementioned antigens.

An infection, disease or disorder which may be treated or prevented by the administration of a vaccine of the invention includes any infection, disease or

disorder wherein a host immune response acts to prevent the infection, disease or disorder. Diseases, disorders, or infection which may be treated or prevented by the administration of the immunostimulatory compositions of the invention include, but are not limited to, any infection, disease or disorder caused by or related to a fungus, parasite, virus, or bacteria, diseases, disorders or infections caused by or related to various agents used in bioterrorism, listeriosis, Ebola virus, SARS, small pox, hepatitis A, hepatitis B, hepatitis C, and hepatitis E, diseases and disorders caused by human rhinovirus, HIV (*e.g.*, HIV-1 and HIV-2), and AIDS, Herpes, polio, foot-and-mouth disease, rabies, diseases or disorders caused by or related to: rotavirus, influenza, coxsackie virus, human papilloma virus, SIV, malaria, cancer, *e.g.*, tumors, human herpes viruses, cytomegalovirus (*esp.* Human), Epstein-Barr virus, Varicella Zoster Virus, hepatitis viruses, such as hepatitis B virus, hepatitis A virus, hepatitis C virus a, paramyxoviruses: Respiratory Syncytial virus, parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18, and the like), flaviviruses (*e.g.* Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus), or influenza virus, *e.g.*, influenza A (*e.g.*, subtypes, hemagglutinin (H) and neuraminidase (N)), influenza B, and influenza C, and diseases or disorders caused by or related to infection by bacterial organisms, including gram-positive and gram-negative bacteria. Examples include, but are not limited to, *Neisseria spp.*, including *N. gonorrhea* and *N. meningitidis*, *Streptococcus spp.*, including *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, *S. mutans*; *Haemophilus spp.*, including *H. influenzae* type B, non typeable *H. influenzae*, *H. ducreyi*; *Moraxella spp.*, including *M. catarrhalis*, also known as *Branhamella catarrhalis*; *Bordetella spp.*, including *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*; *Mycobacterium spp.*, including *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. avium*, *M. paratuberculosis*, *M. smegmatis*; *Legionella spp.*, including *L. pneumophila*; *Escherichia spp.*, including enterotoxigenic *E. coli*, enterohemorrhagic *E. coli*, enteropathogenic *E. coli*; *Vibrio spp.*, including *V. cholera*, *Shigella spp.*, including *S. sonnei*, *S. dysenteriae*, *S. flexnerii*; *Yersinia spp.*, including *Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis*, *Campylobacter spp.*, including *C. jejuni* and *C. coli*; *Salmonella spp.*, including *S. typhi*, *S. paratyphi*, *S. choleraesuis*, *S. enteritidis*; *Listeria spp.*, including *L. monocytogenes*; *Helicobacter spp.*, including *H. pylori*; *Pseudomonas spp.*, including *P. aeruginosa*, *Staphylococcus spp.*, including *S. aureus*, *S. epidermidis*; *Enterococcus spp.*, including *E. faecalis*, *E. faecium*; *Clostridium spp.*, including *C. tetani*, *C. botulinum*, *C. difficile*; *Bacillus spp.*, including *B. anthracis*; *Corynebacterium spp.*, including *C. diphtheriae*; *Borrelia spp.*, including *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. andersonii*, *B. hermsii*; *Ehrlichia spp.*, including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia spp.*, including *R. rickettsii*; *Chlamydia spp.*, including *C. trachomatis*, *C. pneumoniae*, *C.*

*psittaci*; *Leptospira* spp., including *L. interrogans*; *Treponema* spp., including *T. pallidum*, *T. denticola*, *T. hyodysenteriae*. Preferred bacteria include, but are not limited to, *Listeria*, mycobacteria, mycobacteria (e.g., tuberculosis), Anthrax, *Salmonella* and *Listeria monocytogenes*, *Bordetella pertussis*, *Vibrio cholerae*, flukes, mycoplasma, roundworms, tapeworms, *Chlamydia trachomatis*, and malaria parasites,.

As used herein, the term "bacterial infections" include infections with a variety of In another embodiment, T cells can be removed from a patient, and contacted *in vitro* with an anti-GITR binding molecule, optionally with an activating signal (e.g., antigen plus APCs or a polyclonal antibody) and reintroduced into the patient.

Regulatory T cells play an important role in the maintenance of immunological self-tolerance by suppressing immune responses against autoimmune diseases and cancer. Accordingly, in one embodiment, abrogating the suppression of T effector cells by T regulatory cells would be beneficial for enhancing an immune response in cancer. Therefore, the binding molecules of the invention can be used in the treatment of malignancies, to inhibit tumor growth or metastasis. The binding molecules may be administered systemically or locally to the tumor site.

In one embodiment, modulation of GITR function may be useful in the induction of tumor immunity, *i.e.*, for the treatment of a subject with a neoplastic disease or cancer. In one embodiment, a binding molecule of the invention reduces tumor size, inhibits tumor growth and/or prolongs the survival time of a tumor-bearing subject. A GITR binding molecule can be administered to a patient having tumor cells (e.g., sarcoma, melanoma, lymphoma, leukemia, neuroblastoma, carcinoma) to overcome tumor-specific tolerance in the subject.

By the term "tumor-related antigen," as used herein, is meant an antigen which affects tumor growth or metastasis in a host organism. The tumor-related antigen may be an antigen expressed by a tumor cell, or it may be an antigen which is expressed by a non-tumor cell, but which when so expressed, promotes the growth or metastasis of tumor cells. The types of tumor antigens and tumor-related antigens include any known or heretofore unknown tumor antigen, including, without limitation, the bcr/abl antigen in leukemia, HPVE6 and E7 antigens of the oncogenic virus associated with cervical cancer, the MAGE1 and MZ2-E antigens in or associated with melanoma, and the MVC-1 and HER-2 antigens in or associated with breast cancer.

As used herein, the term "neoplastic disease" is characterized by malignant tumor growth or in disease states characterized by benign hyperproliferative and hyperplastic cells. The common medical meaning of the term "neoplasia" refers to "new cell growth" that results as a loss of responsiveness to normal growth controls, e.g., neoplastic cell growth.



As used herein, the terms "hyperproliferative", "hyperplastic", malignant" and "neoplastic" are used interchangeably, and refer to those cells in an abnormal state or condition characterized by rapid proliferation or neoplasia. The terms are meant to include all types of hyperproliferative growth, hyperplastic growth, cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. A "hyperplasia" refers to cells undergoing an abnormally high rate of growth. However, as used herein, the terms neoplasia and hyperplasia can be used interchangeably, as their context will reveal, referring generally to cells experiencing abnormal cell growth rates. Neoplasias and hyperplasias include "tumors," which may be either benign, premalignant or malignant.

The terms "neoplasia," "hyperplasia," and "tumor" are often commonly referred to as "cancer," which is a general name for more than 100 disease that are characterized by uncontrolled, abnormal growth of cells. Examples of cancer include, but are not limited to: breast; colon; non-small cell lung, head and neck; colorectal; lung; prostate; ovary; renal; melanoma; and gastrointestinal (*e.g.*, pancreatic and stomach) cancer; and osteogenic sarcoma.

In one embodiment, the cancer is selected from the group consisting of: pancreatic cancer, melanomas, breast cancer, lung cancer, bronchial cancer, colorectal cancer, prostate cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, cancer of hematological tissues.

Accordingly, this invention also relates to a method of treating neoplastic disease or cancer in a subject, preferably a human, or other animal by administering to such subject or animal an effective amount of a binding molecule of the invention. One skilled in the art is able, by routine experimentation, to determine what an effective amount of polypeptide would be for the purpose of treating neoplastic disease or cancer. For example, a therapeutically effective amount of a binding molecule of the invention may vary according to factors such as the disease stage (*e.g.*, stage I versus stage IV), age, sex, medical complications (*e.g.*, immunosuppressed conditions or diseases) and weight of the subject, and the ability of the binding molecule to elicit a desired response in the subject. The dosage regimen may be adjusted to provide the optimum therapeutic and/or prophylactic response. For example, several divided doses may be administered daily, or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. Generally, however, an effective dosage is expected to be in the



range of about 0.05 to 100 milligrams per kilogram body weight per day and more preferably from about 0.5 to 10, milligrams per kilogram body weight per day.

5

### *Methods of Enhancing Immune Responses*

The subject binding molecules may also be used in methods of enhancing immune responses. Upregulation of immune responses may be in the form of enhancing an existing immune response or eliciting an initial immune response. For example, enhancing an immune response by modulation of GITR may be useful in cases of viral infection. As anti-GITR binding molecules act to enhance immune responses, they would be therapeutically useful in situations where more rapid or thorough clearance of pathogenic agents, *e.g.*, bacteria and viruses would be beneficial. Accordingly, the anti-GITR binding molecules of the invention may be used therapeutically, either or alone or in combination with an antigen or an additional immunostimulatory agent, to treat a subject suffering from a disease or disorder, such as an infectious disease or malignancy, *e.g.*, those listed *supra*.

Anti-GITR binding molecules may also be used prophylactically in vaccines against various pathogens. Immunity against a pathogen, *e.g.*, a virus, could be induced by vaccinating with a viral protein along with a GITR binding molecule (as described above). Alternately, an expression vector which encodes genes for both a pathogenic antigen and a GITR binding molecule, *e.g.*, a vaccinia virus expression vector engineered to express a nucleic acid encoding a viral protein and a nucleic acid encoding a GITR binding molecule, can be used for vaccination. Pathogens for which vaccines may be useful include, for example, hepatitis B, hepatitis C, Epstein-Barr virus, cytomegalovirus, HIV-1, HIV-2, influenza, tuberculosis, malaria and schistosomiasis.

The present invention is further directed to binding molecule-based therapies which involve administering binding molecules of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating, detecting, and/or preventing one or more of the disclosed diseases, disorders, or conditions. Therapeutic compounds of the invention include, but are not limited to, binding molecules of the invention (including analogs and derivatives thereof as described herein) and anti-idiotypic binding molecules as described herein. A binding molecule of the invention can be used to treat, diagnose, inhibit or prevent diseases, disorders or conditions associated with aberrant activity of GITR, including, but not limited to, any one or more of the diseases, disorders, or conditions described herein (*e.g.*, binding molecules of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein).

A binding molecule of this invention may be advantageously utilized in combination with other monoclonal or chimeric binding molecules, or with lymphokines or hematopoietic growth factors (such as, *e.g.*, IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with a binding molecule.

A binding molecule of the invention may be administered alone or in combination with other types of treatments (*e.g.*, radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents, antibiotics, therapy directed against a pathogenic agent (such as for example an immunotherapeutic or chemotherapeutic agent effective against a viral pathogen or a bacterial antigen) and immunostimulatory agents. A binding molecule of the invention may also be administered in combination with an antigen to which an enhanced immune response is desired, *e.g.*, a vaccine or an antigen from a pathogenic agent (or an attenuated form of a virus or bacterium) or an antigen from a tumor as described above. In one embodiment, a binding molecule of the invention is administered alone or in a combination therapy to a subject with an infection. In another embodiment, a binding molecule of the invention is administered alone or in combination to a subject with a chronic viral infection. In yet another embodiment, a binding molecule of the invention are administered alone or in combination to a subject with cancer.

Generally, administration of binding molecules derived a species that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, human binding molecules, derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

## **VI. *Pharmaceutical Compositions***

A binding molecule of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises a binding molecule of the invention and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of

administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable

solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier.

5 They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically  
10 compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant  
15 such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable  
20 propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents,  
25 bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*,  
30 with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, a binding molecule of the invention is prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery  
35 systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations should be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and



Nova Pharmaceuticals, Inc. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

## VII. *Administration of Binding molecules of the Invention*

Binding molecules of the invention are contacted with cells from a subject in a biologically compatible form *in vitro* or *in vivo*. By "biologically compatible form" is meant a form of the agent to be administered in which any toxic effects are outweighed by the therapeutic effects of the binding molecule.

In one embodiment, the subject compositions are administered to a subject. Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of binding molecule may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the binding molecule to elicit a desired response in the individual. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of a binding molecule of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the binding molecule may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the binding molecule to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the binding molecule are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are

dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

5                   An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of a binding molecule of the invention is, *e.g.*, from about 0.1-25 mg/kg, from about 1.0-10 mg/kg, from about 0.5-2.5 mg/kg, from about 5-25mg/kg, from about 1-400 mg/kg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that  
10   for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. Additional, non-limiting ranges for a  
15   therapeutically or prophylactically effective amount of a binding molecule of the invention is from about 0.0001 to 100 mg/kg, and from about 0.01 to 5 mg/kg (*e.g.*, 0.02 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1mg/kg, 2 mg/kg, etc.), of the subject body weight. For example, dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg, preferably at least 1 mg/kg. Doses intermediate in the  
20   above ranges are also intended to be within the scope of the invention.

                  Subjects can be administered such doses daily, on alternative days, weekly or according to any other schedule determined by empirical analysis. An exemplary treatment entails administration in multiple dosages over a prolonged period, for example, of at least six months. Additional exemplary treatment regimes entail  
25   administration once per every two weeks or once a month or once every 3 to 6 months. Exemplary dosage schedules include 1-10 mg/kg or 15 mg/kg on consecutive days, 30 mg/kg on alternate days or 60 mg/kg weekly.

                  Binding molecules of the invention can be administered on multiple occasions. Intervals between single dosages can be, *e.g.*, daily, weekly, monthly or  
30   yearly. Intervals can also be irregular as indicated by measuring blood levels of binding molecule in the patient.

                  Binding molecules of the invention can optionally be administered in combination with other agents that are effective in treating the disorder or condition in need of treatment (*e.g.*, prophylactic or therapeutic). Preferred additional agents are  
35   those which are art recognized and are standardly administered for a particular disorder.

                  The binding molecule can be administered in a convenient manner such as by injection (subcutaneous, intravenous, *etc.*), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of

administration, the active compound can be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. For example, to administer the agent by other than parenteral administration, it may be desirable to coat, or co-administer the agent with, a material to prevent its inactivation.

A binding molecule of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, e.g., *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

In certain embodiments, a binding molecule of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

Binding molecules can be co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Sterna *et al.* (1984) *J. Neuroimmunol.* 7:27).



The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

5           When the active compound is suitably protected, as described above, the binding molecule can be orally administered, for example, with an inert diluent or an assimilable edible carrier.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, a binding molecule of the invention is  
10   coformulated with and/or coadministered with one or more additional therapeutic agents. For example, an anti-GITR binding molecule of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets *e.g.*, antibodies that bind other cytokines or that bind cell surface molecules. Such combination therapies may advantageously utilize lower dosages of the administered  
15   therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

The present invention further encompasses binding molecules conjugated to a diagnostic or therapeutic agent. A binding molecule can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical  
20   testing procedure to, *e.g.*, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting  
25   metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance may be coupled or conjugated either directly to the binding molecule or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Pat. No. 4,741,900 for metal ions which can be conjugated to binding molecules for use as  
30   diagnostics according to the present invention. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a  
35   luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material  $I^{125}$   $I^{131}$ ,  $I^{111}$ ,  $In^{99}$  Tc.

Further, a binding molecule may be conjugated to a therapeutic moiety such as a cytotoxin, *e.g.*, a cytostatic or cytocidal agent, a therapeutic agent, a radioactive metal ion, *e.g.*, alpha-emitters such as, for example,  $^{213}\text{Bi}$ , biological toxins, prodrugs, peptides, proteins, enzymes, viruses, lipids, biological response modifiers, pharmaceutical agents, immunologically active ligands (*e.g.*, lymphokines or other antibodies). In another embodiment, a binding molecule of the invention can be conjugated to a molecule that decreases vascularization of tumors. In other embodiments, the disclosed compositions may comprise binding molecules of the invention coupled to drugs or prodrugs. Still other embodiments of the present invention comprise the use of binding molecules of the invention conjugated to specific biotoxins or their cytotoxic fragments such as ricin, gelonin, pseudomonas exotoxin or diphtheria toxin. The selection of which conjugated or unconjugated binding molecule to use will depend on the type and stage of cancer, use of adjunct treatment (*e.g.*, chemotherapy or external radiation) and patient condition. It will be appreciated that one skilled in the art could readily make such a selection in view of the teachings herein..

A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

This invention is further illustrated by the following examples, which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures, are incorporated herein by reference.

**EXAMPLES**

The following materials and methods were used in certain Examples:

5    ***Methods****Culture of T cell lines*

Differentiated cell lines were produced from cells prepared from human cord blood or peripheral blood CD4+CD45RA+ naïve T cells by a variety of methods, including flow cytometry and magnetic bead separations. Purity of the starting  
 10    populations was >95%. Cells were then stimulated by CD3 and CD28 antibodies in RPMI 1640 with 10%FCS and 1% Human AB serum with defined mixtures of cytokines and neutralizing antibodies to cytokines to produce the differentiated cell types. Th1 cells were produced by culture with IL12 (62U/ml) and anti-IL4 (0.2 µg/ml); Th2 cells were produced by culture in IL4 (145U/ml) and anti-IL12 (10ug/ml) and anti-  
 15    IFNγ (10ug/ml); and regulatory T cells were produced by culture in TGFβ (32U/ml), IL9 (42U/ml), anti-IL4 (10ug/ml) and anti-IL12 (10ug/ml) and anti-IFNγ(10ug/ml). (Note: anti-IL12 was not used in all experiments). All cultures were supplemented with IL2 (65U/ml) and IL15 (4500U/ml). Cells were split into larger culture dishes as warranted by cell division.

20

**EXAMPLE 1: Isolation and Purification of 6C8**

The 6C8 antibody is an IgG2b, kappa. Purification of this antibody revealed the presence of a double heavy chain (Figure 1). This could have been due to alternative glycosylation or contamination with another Ab. Size exclusion  
 25    chromatography showed the presence of one peak (Figure 2).

The 6C8 antibody was purified as follows:

1.       Washed 20 ml Protein G (Pharmacia HR 10/30) with 5CV of dPBS
2.       Loaded 1L (run 1) or 2 L (run 2) of hGITR (6C8) supernatant
- 30       3.       Washed with 10 CV of dPBS
4.       Eluted with 100 mM Citrate, pH 2.8 directly into 1 M Tris (20-25% v: v)
5.       Stripped with 100 mM Citrate, pH 2.8, 0.3 M NaCl

35    **EXAMPLE 2: Characterization of 6C8**

The 6C8 antibody binds to GITR-L-M transfected cells (Figure 3) and activated PBLs (Figure 4). The saturation curve of biotin-labeled anti-GITR on activated lymphocytes suggests a good relative affinity (Figure 5).

The 6C8 antibody is co-stimulatory on T lymphocytes activated with suboptimal anti-CD3 (Figure 6). This antibody does not co-stimulate to the same level as CD28, but it is comparable to the commercial anti-GITR (R&D).

5 The 6C8 antibody does not induce apoptosis on activated lymphocytes (Figure 7). Lymphocytes were activated with PHA for 3 days prior to the addition of the antibody. Compared to YTH 655 (anti-human CD2 known to induce apoptosis on activated lymphocytes) 6C8 does not increase the apoptosis of activated T lymphocytes.

The 6C8 antibody does not block a primary mixed lymphocyte reaction (MLR) (Figure 8). TRX1 (anti-human CD4) was used as a positive control for the MLR.

10

### **EXAMPLE 3: The 6C8 Antibody Abrogates Suppression of T Effector Cells Induced by T Regulatory Cells**

The 6C8 antibody was able to block the suppression induced by T regulatory cells (Figure 9). CD4+/CD25+ cells were added to CD4+/CD25- cells at  
15 various ratios. The cells were stimulated with plate-bound anti-CD3 and anti-CD28. At a ratio of 1:1 the CD4+/CD25+ cells were able to abrogate the proliferation of the CD4+/CD25- cells. The addition of 6C8 to the cultures was able to block the suppression in a dose-dependent manner.

20 When T cells were stimulated through anti-CD3 only (with no co-stimulation with anti-CD28) there was no suppression observed with the addition of CD4+/CD25+ cells to the CD4+/CD25- cells, in fact, the anti-GITR antibody was slightly co-stimulatory under these conditions (Figure 10).

### **EXAMPLE 4: The 6C8 Antibody Modulates Signaling via NF- $\kappa$ B**

25 Activation of T cells *via* CD3 or CD3 and CD28 results in activation of I- $\kappa$ B signaling pathways, as assessed by both I- $\kappa$ B phosphorylation (Fig.12 and 14) and subsequent degradation (Fig.11 and 13 ).

As presented on Fig. 11, under conditions of partial activation, anti-GITR has a significant effect on I- $\kappa$ B signaling, as assessed by time dependent degradation of  
30 I- $\kappa$ B. In the presence of the GITR binding molecule, degradation is significantly attenuated, at all time points analyzed. Above changes nicely correlate with decline of phosphorylation of I- $\kappa$ B (Fig.12).

Interestingly the magnitude of response is greater for TH2 and Treg vs. TH1. Furthermore the expression of GITR appears to be higher on TH1 cells, compared  
35 to TH2 and Treg cells, (as assessed by MCF (mean channel fluorescence)) in parallel experiments. T cells fully activated *via* crosslinking to CD3 and CD28 lose their responsiveness to anti-GITR, however fully retain activation of I- $\kappa$ B *via* TNF- $\alpha$ .



**EXAMPLE 5: The 6C8 Antibody Enhances Immune Responses**

The B16 melanoma tumor model is an aggressive melanoma model that has been used to study the role of T regulatory cells in cancer. Treatment of mice with a depleting anti-CD25 antibody or anti-CTLA-4 has shown promising results in this model. In both cases, treatments were able to delay tumor on-set and tumor size. Since GITR is expressed on CD25+ cells and may be involved in abrogating the suppression of T regulatory cells, B16-tumor bearing mice were treated with anti-GITR binding molecule to determine if there was an effect on tumor on-set or tumor size. Treatment with anti-GITR binding molecule one day after the mice were injected with tumor resulted in a delayed onset and size of tumor (Figure 17). In addition, there were still mice in the GITR treated group that were tumor-free at the end of the study.

All animals were injected with  $10^4$  B16 melanoma cells in their right flank on day 0. The GITR groups received 2 milligrams, 1 milligram, 0.5, milligrams, or 0.2 milligrams of anti-GITR binding molecule on Day 1. Measurable tumors were visible starting on Day 16.

**EXAMPLE 6: Simultaneous Delivery of Anti-GITR and Antigen Results in An Adjuvant Effect**

The adjuvant effect of an anti-mGITR antibody on the humoral response to ovalbumin (Ova) or hemagglutinin (HA) was further investigated. Mice were treated with either no antibody, YAML (isotype control), or 2F8 (rat-anti-mGITR) on days -1, 0, and 1 at 0.4 mg/day. To assess the importance of Fc receptor engagement in the mechanism of action of the binding molecule, an additional group of animals was treated with 6 mg/day of 2F8 F(ab')<sub>2</sub> on days -1, 0 and 1. This dose was selected based on the short half life of F(ab')<sub>2</sub> compared to whole antibody. Mice were immunized with Ova (100µg) or HA (10 µg) on day 0. The Ova treated mice were challenged with 100 µg Ova on day 14 and then bled on days 21 and 28 to obtain serum samples for ELISA assays. HA treated mice were challenged with 5 µg HA on day 14 and also bled on days 21 and 28.

Serum concentrations of 2F8 and 2F8 F(ab')<sub>2</sub> were monitored to assess the pharmacokinetic profiles of the binding molecules. On day 1, serum levels of binding molecule in mice treated with 2F8 or the 2F8 F(ab')<sub>2</sub> fragments were comparable. Binding molecule was detected in the 2F8 treated mice until day 9, whereas the 2F8 F(ab')<sub>2</sub> fragment treated mice had detectable binding molecule only until day 3, despite a 15X higher dose.

The results demonstrate that in the HA arm of the study, mice treated with 2F8 had a 4 and 5 fold increase in anti-HA antibodies compared to animals treated with no antibody and an 18 and 20 fold increase in anti-HA antibodies compared to

YAML treated mice on days 21 and 28, respectively (Figure 19). The anti-HA titer observed with the anti-mGITR antibody as an adjuvant is comparable to the titer observed when HA was administered with Incomplete Freund's adjuvant (IFA). This suggests that the response observed with the anti-mGITR antibody is comparable to one of the most potent adjuvants frequently utilized in immunological studies.

In the Ova arm of the study, mice treated with 2F8 had a 13 and 6 fold increase in anti-Ova antibodies compared to animals treated with no antibody and a 17 and 8 fold increase in anti-Ova antibodies compared to YAML treated mice on day 21 and day 28, respectively (Figure 20). The effect of the 2F8 antibody on the response to Ova was comparable to the observed response to HA. Mice treated with 2F8 F(ab')<sub>2</sub> had a 4 and 3 fold increase in Anti-Ova antibodies compared to animals treated with no antibody and a 6 and 5 fold increase in anti-Ova antibodies compared to YAML treated mice on day 21 and day 28, respectively (Figure 20). The dose of F(ab')<sub>2</sub> and the different pharmacokinetic profile compared to whole antibody may explain the decreased anti-Ova response when compared to the 2F8 treated mice.

Together, these data demonstrate that the effect of the 2F8 antibody on the humoral response to antigen is predominantly attributable to the F(ab')<sub>2</sub> portion of the antibody and that Fc receptor engagement may not be required for the adjuvant effect of the anti-mGITR antibody.

20

#### **EXAMPLE 7: Preparation of a Chimeric anti-GITR Binding Molecule**

The 6C8 variable light chain region was grafted to a human light chain constant region using conventional molecular biological techniques. The IgG1 light chain constant region was used. The amino acid sequence of the complete chimeric light chain GITR binding molecule is shown below:

25

DIVMTQSQKFMSTSVGDRVSVTCKASQNVGTNVAWYQQKPGQSPKALIYSASY  
RYSQVPDRFTGSGSGTDFTLTINNVSIEDLAIEYFCQQYNTDPLTFGAGTKLEIKR  
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQES  
VTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC  
(SEQ ID NO:22).

30

The 6C8 variable heavy chain was also grafted to a human heavy chain constant region using conventional molecular biological techniques. The IgG1 heavy chain constant region was used. The amino acid sequence of the complete chimeric heavy chain GITR binding molecule is shown below (also referred to as "Gly"):

QVTLKESGPGILKPSQTLSTCSFSGFSLSTSGMGVGVWIRQPSGKGLEWLAHIW  
WDDDKYYNPSLKSQLTISKDTSRNQVFLKITSVDTADAATYYCARTRRYFPFAY  
WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAAALGCLVKDYFPEPVTVSWNS

35

GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHNKPSNTKVDDK  
 VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE  
 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK  
 CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS  
 5 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVM  
 HEALHNHYTQKSLSLSPGK (SEQ ID NO:23).

Since the amino acid sequence NX(S/T) is a putative consensus sequence  
 for a glycosylation site which may affect the production of the binding molecule, and  
 10 IgG1 constant region of the 6C8 heavy chain has the sequence NST, a second version of  
 the heavy chain constant region was prepared to conservatively substitute a glutamine  
 for an asparagine at amino acid residue 299 (bolded and underlined above) of SEQ ID  
 NO:23. Accordingly, a second human constant region was grafted to the 6C8 heavy  
 chain variable region. The amino acid sequence of the complete chimeric heavy chain  
 15 GITR binding molecule is shown below (also referred to a "Agly"):  
 QVTLESGPGILKPSQTLSTCSFSGFSLSTSGMGVGWIRQPSGKGLEWLAHIW  
 WDDDKYYNPSLKSQTLTISKDTSRNQVFLKITSVDTADAATYYCARTRRYFPFAY  
 WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS  
 GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHNKPSNTKVDDK  
 20 VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE  
 DPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYK  
 CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS  
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVM  
 HEALHNHYTQKSLSLSPGK (SEQ ID NO:24).

25

#### **EXAMPLE 8: Preparation of Humanized Forms of the 6C8 Anti-GITR Binding Molecule**

The CDR homology based strategy described in Hwang *et al.* (2005)  
*Methods* (36) 35-42 was used to humanize 6C8. The heavy and light chain amino acid  
 30 sequences were blasted using a publicly available database, and the results indicated that  
 6C8 had a 3-1 heavy chain canonical structure and a 2-1-1 light chain canonical  
 structure. From this, all germ line kappa chain V genes with a 2-1-1 canonical structure  
 in the IMGT database were compared with the 6C8 antibody sequence. The same was  
 done for the heavy chain where all 3-1 germ line heavy chain V genes were compared to  
 35 the 6C8 amino acid sequence. Only the CDR sequences were compared and the  
 frameworks were selected based on which germline sequences had the most matches in  
 the CDRs. (see alignments below).

For the light chain, the 3-15\*01 sequence had 14 matches in the CDRs and was selected. Since CDR 3 ends with leucine and threonine, the Jk4 J gene segment sequence was used.

### Light Chain V Genes with 2-1-1 Canonical Structure

5

IMGT

Gene Name	CDR1	CDR2	CDR3	IDs
IGKV1-5	RASQSISWLA.....	DASSLES.....	QQYNSYS..	11
10 IGKV1-6	RASQGIRNDLG.....	AASSLSQ.....	LQDYNYP..	9
IGKV1-9	RASQGISSYLA.....	AASTLQS.....	QQLNSYP..	11
IGKV1-12	RASQGISSWLA.....	AASSLQS.....	QQANSFP..	11
IGKV1-16	RASQGISSWLA.....	AASSLQS.....	QQYNSYP..	12
IGKV1D-16	RARQGISSWLA.....	AASSLQS.....	QQYNSYP..	11
15 IGKV1-17	RASQGIRNDLG.....	AASSLQS.....	LQHNSYP..	9
IGKV1-27	RASQGISNYLA.....	AASTLQS.....	QKYN SAP..	11
IGKV1-33	QASQDISNYLN.....	DASNLET.....	QQYDNLP..	9
IGKV1-39	RASQSISSYLN.....	AASSLQS.....	QQSYSTP..	9
IGKV1D-43	WASQGISSYLA.....	YASSLQS.....	QQYYSTP..	11
20 IGKV3-11	RASQSVSSYLA.....	DASNRAT.....	QQRSNWP..	11
IGKV3D-11	RASQGVSSYLA.....	DASNRAT.....	QQRSNWH..	10
IGKV3-15	RASQSVSSNLA.....	GASTRAT.....	QQYNNWP..	14
6C8	KASQNVGTNVA.....	SASYRYS.....	QQYNTDP	

25

All germ line light chain kappa chain V genes with a 2-1-1 canonical structure in the IMGT database were compared with the 6C8 antibody sequence. The same was done for the heavy chain where all 3-1 germ line heavy chain V genes were compared to the 6C8 amino acid sequence

30

Using this methodology one version of the light chain was made:

EIVMTQSPATLSVSPGERATLSCKASQNVGTNVAWYQQKPGQAPRLLIYSASYRYS  
GIPARFSGSGSGTEFTLTISSLQSEDAVYYCQQYNTDPLTFGGGGTKVEIK (SEQ  
ID NO:44) (the CDRs are italicized)

35

For the heavy chain, sequence 2-05\*01 had 17 matches. However, the sequences around CDR 3 were different than 6C8 (YYCAR vs. YYCAHR). Since CDR



3 has been shown to be the most important CDR for recognition, it is important to keep this area as perfectly matched as possible. Sequence 2-70\*01 had 16 matches in the CDRs and the sequences right before CDR 3 perfectly matched 6C8's and so 2-70\*01 was selected.

5                   For the J gene segment of the heavy chain, JH4 had the most matches and was therefore, selected. The amino acid sequences were then reverse translated and primers corresponding to the desired nucleotide sequence were obtained from IDT (Coralville, IA).

10   **Heavy Chain V Genes with 3-1 Canonical Structures**

IMGT			
Gene Name	CDR1	CDR2	IDs
IGHV2-5	TSGVGVG.....	LIYWNDDKRYSPSLKS	17
IGHV2-26	NARMGVS.....	HIFSNDEKSYSTSLKS	12
IGHV2-70	TSGMCVS.....	LIDWDDDKYYSTSLKT	16
IGHV4-30-2	SGGYSWS.....	YIYHSGSTYYNPSLKS	10
IGHV4-30-4	SGDYYWS.....	YIYYSGSTYYNPSLKS	9
IGHV4-31	SGGYYSWS.....	YIYYSGSTYYNPSLKS	9
IGHV4-39	SSSYWWG.....	SIYYSGSTYYNPSLKS	10
IGHV4-61	SGSYYWS.....	YIYYSGSTNYNPSLKS	8
6C8	TSGMGVG.....	HIWWDDDKYYNPSLKS	

25

Using this methodology one version of the heavy chain was made:

QVTLRSGPALVKPTQTLTLTCTFSGFSLSTSGMGVGVWIRQPPGKALEWLAHIWW  
DDDKYYNPSLKSRILTISKDTSKNQVVLTMNMDPVDATYYCARTRRYFPFAYW  
30 GQGTLVTVSS (SEQ ID NO:53) (also referred to as "N")

35                   Since the amino acid sequence NX(S/T) is a putative consensus sequence for a glycosylation site which may affect the production of the binding molecule, and CDR2 of the 6C8 heavy chain has the sequence NPS, a second version of the heavy chain was prepared to conservatively substitute a glutamine for an asparagine at amino acid residue 62 (bolded and underlined above) of SEQ ID NO:53. Accordingly, a second heavy chain version was made:

QVTLRESGPALVKPTQTLTLTCTFSGFSLSSTSGMGVGVWIRQPPGKALEWLAHIWW  
 DDDKYYQPSLKSRLTISKDTSKNQVVLTMNTMDPVDATYYCARTRRYFPFAYW  
 GQGTLVTVSS (SEQ ID NO:54) (also referred to as "Q").

5

A CLUSTAL W (1.82) multiple sequence alignment (using a Blosum scoring matrix with a gap penalty of 10) of the 6C8 light chain variable region and the 3-15\*01 germline light chain sequence was also performed. The results are presented below:

10

```

6C8          DIVMTQSQKFMSTSVGDRVSVTCKASQNVGTNVAWYQQKPGQSPKALIYSASYRYSQVDP
3-15*01      EIVMTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIPA
:*****      :*. * *:*.::*:***.*.:*:*****:*. :***.* * :*:*
```

15

```

6C8          RFTGSGSGTDFTLTINNVSIEDLAIEYFCQYNTDPLTFGAGTKLEIK
3-15*01      RFSGSGSGTEFTLTISSLQSEDFAVYYCQYNNWP-----
*:*****:*****.:*:***:* *:*****. *
```

20

Based on the CLUSTAL W analyses, several amino acid residues in the human framework were identified for potential substitution with amino acid residues corresponding to the 6C8 framework residues in the humanized 6C8 light chain.

Specifically, the E at position 1, the P at position 8, the A at position 9, the T at position 10, the L at position 11, the V at position 13, the P at position 15, the E at position 17, the A at position 19, the T at position 20, the L at position 21, the S at position 22, the A at position 43, the R at position 45, the L at position 46, the I at position 58, the A at position 60, the S at position 63, the E at position 70, the S at position 76, the S at position 77, the L at position 78, the Q at position 79, the F at position 83, the V at position 85, the Y at position 87, the G at position 100, and the V at position 104.

30

Similarly, a CLUSTAL W (1.82) multiple sequence alignment (using a Blosum scoring matrix with a gap penalty of 10) of the 6C8 heavy chain variable region and the germline heavy chain proteins with a 2-70\*01 amino acid sequence was also performed. The results are presented below:

35

```

40  6C8          QVTLKESGPGILKPSQTLSTLCTFSGFSLSSTSGMGVGVWIRQPSGKGLEWLAHIWWDDDKY
    2-70*01      QVTLRESGPALVKPTQTLTLTCTFSGFSLSSTSGMCSVWIRQPPGKALEWLALIDWDDKY
    *****:*****.:*:***:***:*****:*****.*****.* *****
```

```

    6C8          YNP SLKSQLTISKDTSRNQVFLKITSVDATAATYYCARTRRYFPFAYWGQGTLVTVSS
    2-70*01      YSTSLKTRLTISKDTSKNQVVLTMNTMDPVDATYYCARI-----
```

45

```

*:*****:*****:***.*.:*.:*.:*.:*****
```

Based on the CLUSTAL W analyses, several amino acid residues in the human framework were identified for potential substitution with amino acid residues corresponding to the 6C8 framework residues in the humanized 6C8 heavy chain. Specifically, the R at position 5, the A at position 10, the L at position 11, the V at position 12, the T at position 15, the T at position 19, the T at position 23, the P at position 43, the A at position 46, the R at position 68, the K at position 77, the V at position 81, the T at position 83, the M at position 84, the N at position 86, the M at position 87, the P at position 89, the V at position 90, and/or the T at position 92.

Four humanized full-length 6C8 binding molecules were made having the following humanized heavy and light chain combinations:

Full-length Version 1 (HuN6C8-Gly) – humanized (Hu) 6C8 Light chain (L)/humanized Heavy chain with the N in CDR2 (“N”) and comprising a constant region having an N (“Gly”)

Full-length Version 2 (HuN6C8-Agly) – humanized (Hu) 6C8 Light chain (L)/humanized Heavy chain with the N in CDR2 (“N”) and comprising a constant region having an A (“Agly”)

Full-length Version 3 – (HuQ6C8-Gly)– humanized (Hu) 6C8 Light chain (L)/humanized Heavy chain with the Q in CDR2 (“Q”) and comprising a constant region having an N (“Gly”)

Full-length Version 4 – (HuQ6C8-Agly)– humanized (Hu) 6C8 Light chain (L)/humanized Heavy chain with the Q in CDR2 (“Q”) and comprising a constant region having an A (“Agly”)

The amino acid sequence of the glycosylated IgG1 heavy chain constant region that was used to make the full-length binding molecules is shown below:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA  
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT  
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD  
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE  
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE  
NNYKTTTPVLDSFGSFLYSKLTVDKSRWQQGNVVFSCSVMHEALHNHYTQKSL  
SLSPGK (SEQ ID NO:55).

The amino acid sequence of the aglycosylated IgG1 heavy chain constant region that was used to make the full-length binding molecules is shown below:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA  
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT  
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD

GVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE  
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE  
NNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVCSVMHEALHNHYTQKSL  
SLSPGK (SEQ ID NO:56).

- 5                   The amino acid sequence of the IgG1 light chain constant region that was  
used to make the full-length binding molecules is shown below:

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC  
(SEQ ID NO:57).

10

                  The complete amino acid sequence of the humanized 6C8 light chain is  
shown below:

- EIVMTQSPATLSVSPGERATLSCASQNVGTNVAWYQQKPGQAPRLLIYSASYR  
YSGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNTDPLTFGGGTKVEIKRTV  
15 AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT  
EQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ  
ID NO:58).

The leader sequence METQSQVFVYMLLWLSGVDG (SEQ ID NO:59) may  
optionally be included.

20

                  The complete amino acid sequences of the humanized 6C8 heavy chain  
versions HuN6C8-Agly, HuQ6C8-Gly, and HuQ6C8-Agly are shown below:

HuN6C8-Gly

- 25 QVTLRESGPALVKPTQTLTLTCTFSGFSLSTSGMGVGVWIRQPPGKALEWLAHIW  
WDDDKYYNPSLKSRLTISKDTSKNQVVLTMNMDPVDATATYYCARTRRYFPFA  
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN  
SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKK  
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHE  
30 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK  
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HEALHNHYTQKSLSLSPGK (SEQ ID NO:60);

35 HuN6C8-Agly

QVTLRESGPALVKPTQTLTLTCTFSGFSLSTSGMGVGVWIRQPPGKALEWLAHIW  
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SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHNKPSNTKVDDKK  
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 5 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVDFSCSVM  
 HEALHNHYTQKSLSLSPGK (SEQ ID NO:61);

#### HuQ6C8-Gly

QVTLRSGPALVKPTQTLTLTCTFSGFSLSTSGMGVGVWIRQPPGKALEWLAHIW  
 10 WDDDKYYQPSLKSRLTISKDTSKNQVVLMTNMDPVDATYYCARTRRYFPFA  
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 15 CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS  
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVDFSCSVM  
 HEALHNHYTQKSLSLSPGK (SEQ ID NO:62); and

#### HuQ6C8-Agly

QVTLRSGPALVKPTQTLTLTCTFSGFSLSTSGMGVGVWIRQPPGKALEWLAHIW  
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 HEALHNHYTQKSLSLSPGK (SEQ ID NO:63).

30 The leader sequence MDRLTFSFLLLIVPAYVLS (SEQ ID NO:64) may optionally be included.

#### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no  
 35 more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A GITR binding molecule comprising amino acid residues 20-138 of SEQ ID NO.:1 or amino acid residues 20-138 of SEQ ID NO:66.
2. A GITR binding molecule comprising amino acid residues 21-127 of SEQ ID NO.:2.
3. A GITR binding molecule comprising at least one complementarity determining region (CDR) amino acid sequence selected from the group consisting of: SEQ ID NO.:3, SEQ ID NO.:4 or SEQ ID NO:19, and SEQ ID NO.:5.
4. The GITR binding molecule of claim 3, comprising at least 2 CDRs.
5. The GITR binding molecule of claim 3, comprising three CDRs.
6. A GITR binding molecule comprising at least one CDR amino acid sequence selected from the group consisting of: SEQ ID NO.:6, SEQ ID NO.:7, and SEQ ID NO.:8.
7. The GITR binding molecule of claim 6, comprising at least 2 CDRs.
8. The GITR binding molecule of claim 6, comprising three CDRs.
9. A GITR binding molecule comprising the CDRs shown in SEQ ID NOs.: 3, 4 or 19, 5, 6, 7 or 8.
10. A GITR binding molecule comprising a heavy chain variable region comprising amino acid residues 20-138 of SEQ ID NO.:1 or amino acid residues 20-138 of SEQ ID NO:66 and further comprising a light chain variable region comprising the amino acid residues 21-127 of SEQ ID NO.:2.
11. The GITR binding molecule of any one of claims 3-10 comprising human germline heavy and light chain framework regions.

12. The GITR binding molecule of claim 11, wherein one or more human framework amino acid residues is backmutated to the corresponding murine amino acid residue.
- 5 13. The GITR binding molecule of claim 9, wherein the constant region comprises an IgG2b heavy chain constant region.
14. The GITR binding molecule of claim 9, wherein the binding molecule binds to human GITR.
- 10 15. The GITR binding molecule of claim 9, wherein the binding molecule does not induce apoptosis.
16. The GITR binding molecule of claim 9, wherein the binding molecule does not block the primary mixed lymphocyte reaction.
- 15 17. The GITR binding molecule of claim 9, wherein the binding molecule abrogates the suppression of T effector cells by T regulatory cells.
- 20 18. The GITR binding molecule of claim 9, wherein the binding molecule enhances effector T cell proliferation.
19. The GITR binding molecule of claim 9, wherein the binding molecule is murine.
- 25 20. The GITR binding molecule of claim 19, wherein said binding molecule comprises a murine IgG2a heavy chain.
21. The GITR binding molecule of claim 9, wherein the binding molecule modulates the activity of human GITR.
- 30 22. The GITR binding molecule of claim 9, wherein the binding molecule attenuates degradation of I- $\kappa$ B in T cells.
- 35 23. The GITR binding molecule of claim 11, wherein said binding molecule is a chimeric antibody.

24. A GITR binding molecule that binds to GITR on human T cells and human dendritic cells and has a binding constant ( $K_d$ ) of  $1 \times 10^{-9}$  or less.

25. The GITR binding molecule of claim 24, wherein the binding  
5 molecule abrogates the suppression of T effector cells by T regulatory cells.

26. The GITR binding molecule of claim 24, wherein said binding molecule is a humanized antibody.

10 27. A composition comprising the GITR binding molecule of any one of claims 1-10 or 13-26 and a pharmaceutically acceptable carrier.

28. The composition of claim 27, further comprising at least one additional therapeutic agent for treating cancer in a subject.

15 29. The composition of claim 27, further comprising at least one additional therapeutic agent for treating a viral infection in a subject.

30. The composition of claim 27, further comprising at least one  
20 tumor antigen for treating cancer in a subject.

31. The composition of claim 27, further comprising at least one antigen from a pathogenic agent for treating a viral infection in a subject.

25 32. A method for abrogating the suppression of T effector cells by T regulatory cells, comprising contacting human immune cells with the GITR binding molecule of any one of claims 1-10 or 13, such that the suppression of T effector cells by T regulatory cells is abrogated.

30 33. A method for modulating T cell receptor induced signaling in an effector T cell, comprising contacting a cell with the GITR binding molecule of any one of claims 1-10 or 13, such that T cell receptor induced signaling in an effector T cell is modulated.

35 34. The method of claim 33, wherein degradation of I- $\kappa$ B is modulated.

35. The method of claim 33, wherein the T cell is a Th1 cell.



36. The method of claim 35, wherein the T cell is a CD4<sup>+</sup> cell.
37. The method of claim 35, wherein the T cell is a CD8<sup>+</sup> cell.
- 5 38. A method for enhancing an immune response in a subject, comprising contacting a cell with the GITR binding molecule of any one of claims 1-10 or 13-26 such that that an immune response in a subject is enhanced.
- 10 39. A method for treating cancer in a subject, comprising contacting a cell with the GITR binding molecule of any one of claims 1-10 or 13-26 such that cancer is treated in a subject.
- 15 40. The method of claim 39, wherein the type of cancer is selected from the group consisting of: pancreatic cancer, melanoma, breast cancer, lung cancer, bronchial cancer, colorectal cancer, prostate cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, and cancer of hematological tissues.
- 20 41. A method for treating an infection, disorder, or disorder caused by a pathogenic agent in a subject, comprising contacting a cell with the GITR binding molecule of claim 1, such that the disease, disorder, or condition caused by a pathogenic agent is treated in a subject.
- 25 42. The method of claim 41, wherein the pathogenic agent is a virus selected from the group consisting of: HIV, human herpes viruses, cytomegalovirus, Rotavirus, Epstein-Barr virus, Varicella Zoster Virus, hepatitis viruses, such as hepatitis B virus, hepatitis A virus, hepatitis C virus and hepatitis E virus, paramyxoviruses: Respiratory Syncytial virus, parainfluenza virus, measles virus, mumps virus, human papilloma viruses, flaviviruses and influenza virus.
- 30 43. The method of claim 41, wherein the pathogenic agent is a bacterium selected from the group consisting of: *Neisseria spp*, *Streptococcus spp*, *S. mutans*, *Haemophilus spp.*, *Moraxella spp*, *Bordetella spp*, *Mycobacterium spp*,
- 35

*Legionella spp, Escherichia spp, Vibrio spp, Yersinia spp, Campylobacter spp, Salmonella spp, Listeria spp., Helicobacter spp, Pseudomonas spp, Staphylococcus spp., Enterococcus spp, Clostridium spp., Bacillus spp, Corynebacterium spp., Borrelia spp., Ehrlichia spp, Rickettsia spp, Chlamydia spp., Leptospira spp., Treponema spp.*

5                    44.     A method for modulating GITR function, comprising contacting human GITR with the GITR binding molecule of any one of claims 1-10 or 13-26 in the presence of a stimulating agent such that GITR function is modulated.

                    45.     An isolated nucleic acid molecule comprising a nucleotide  
10     sequence encoding a heavy chain variable region comprising nucleotides 58-414 of SEQ ID NO.:9.

                    46.     An isolated nucleic acid molecule comprising a nucleotide  
                    sequence encoding a light chain variable region comprising nucleotides 61-381 of SEQ  
15     ID NO.:10.

                    47.     An isolated nucleic acid molecule comprising a nucleotide  
                    sequence encoding at least one CDR selected from the group consisting of: SEQ ID  
                    NO.:11, SEQ ID NO.:12 or SEQ ID NO:65, and SEQ ID NO.:13.  
20

                    48.     The isolated nucleic acid molecule of claim 47, comprising a  
                    nucleotide sequence encoding at least two CDRs.

                    49.     The isolated nucleic acid molecule of claim 47, comprising a  
25     nucleotide sequence encoding three CDRs.

                    50.     An isolated nucleic acid molecule comprising a nucleotide  
                    sequence encoding at least one CDR selected from the group consisting of: SEQ ID  
                    NO.:14, SEQ ID NO.:15 and SEQ ID NO.:16.  
30

                    51.     The isolated nucleic acid molecule of claim 50, comprising a  
                    nucleotide sequence encoding at least two CDRs.

                    52.     The isolated nucleic acid molecule of claim 50, comprising a  
35     nucleotide sequence encoding three CDRs.

                    53.     An isolated nucleic acid molecule comprising the nucleotide  
                    sequences shown in SEQ ID NOs.: 11, 12 or 65, 13, 14, 15, and 16.

54. A recombinant expression vector comprising the nucleic acid molecules of any one of claims 45-53.

5 55. A recombinant expression vector comprising a nucleotide molecule having a nucleotide sequence encoding the binding molecule of claim 24.

56. A host cell into which the recombinant expression vector of claim 55 has been introduced.

10

57. A method for producing a GPCR binding molecule that binds human GPCR, comprising culturing the host cell of claim 56 in a culture medium until a binding molecule that binds human GPCR is produced by the cell.

15

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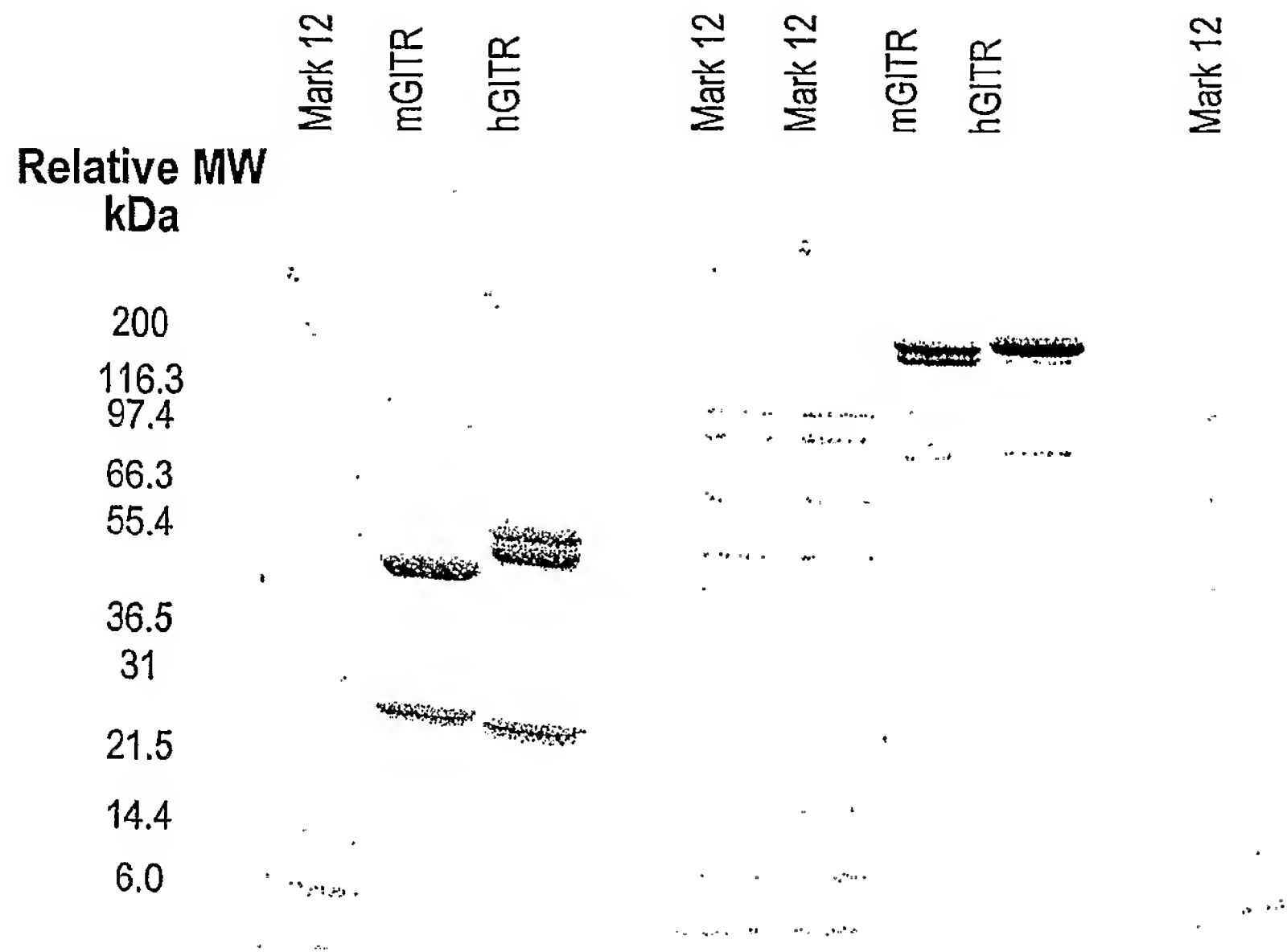


Fig. 1

Mouse  $\alpha$  Human GITR (6C8)

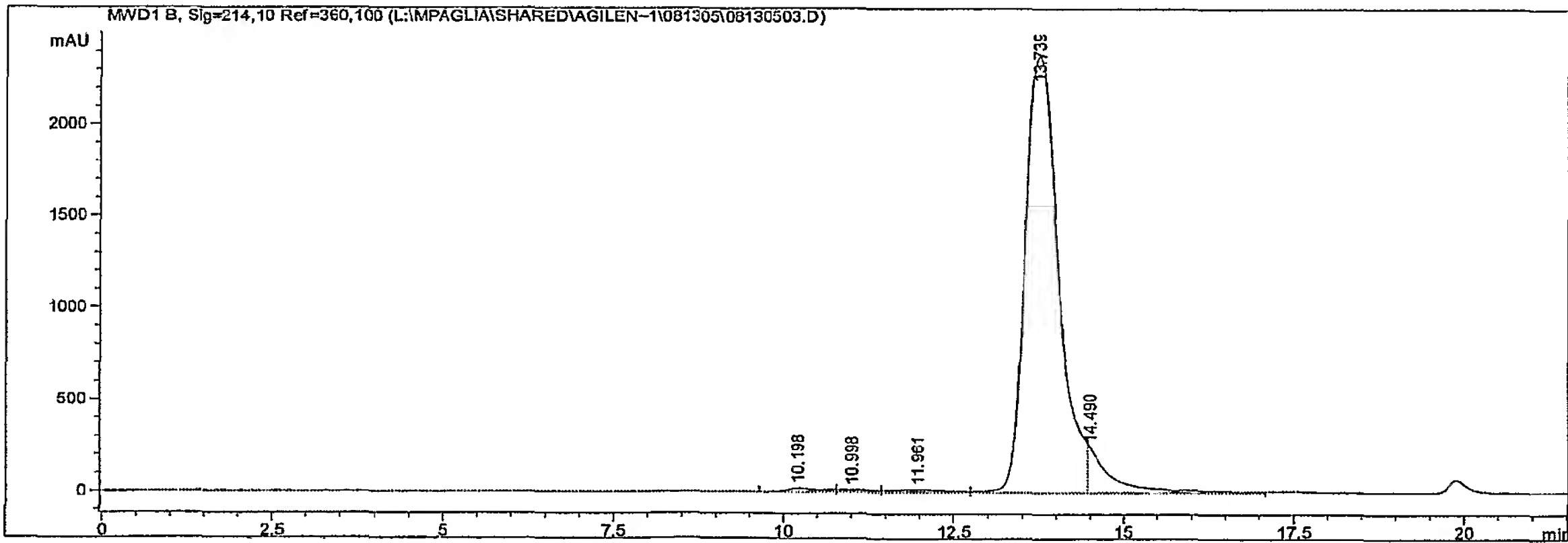


Fig. 2



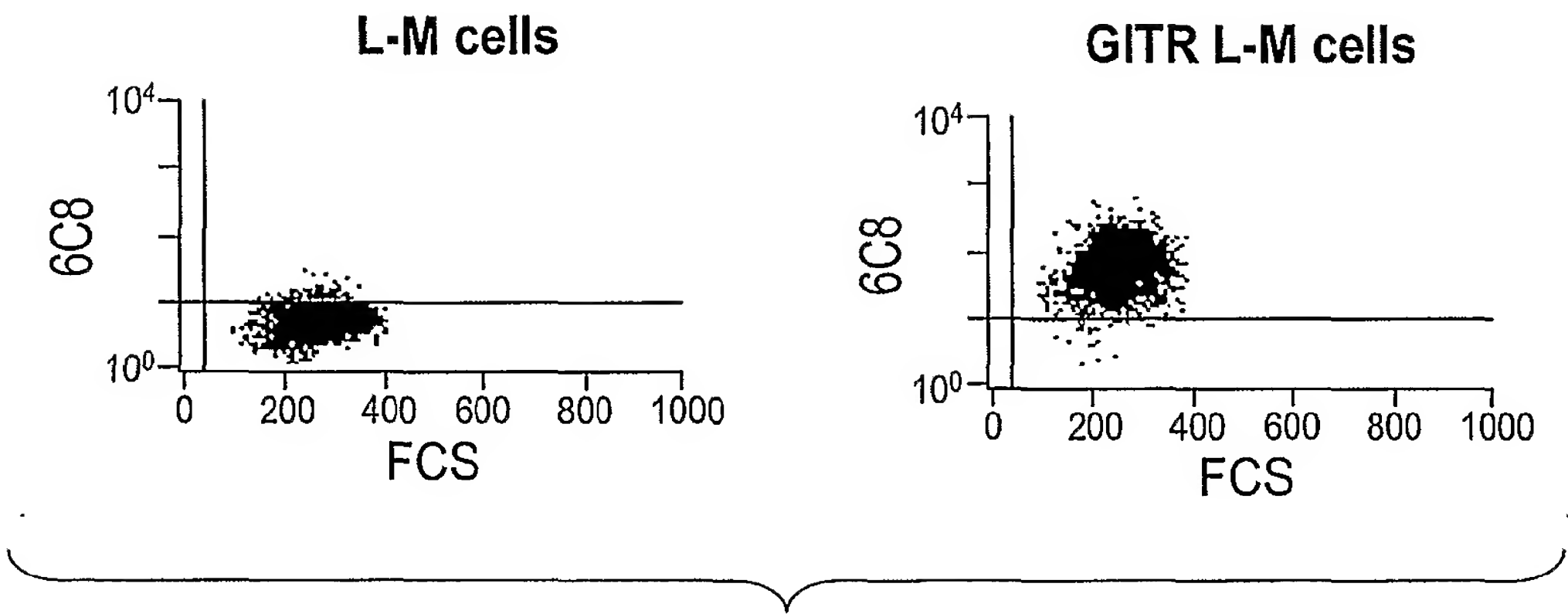


Fig. 3

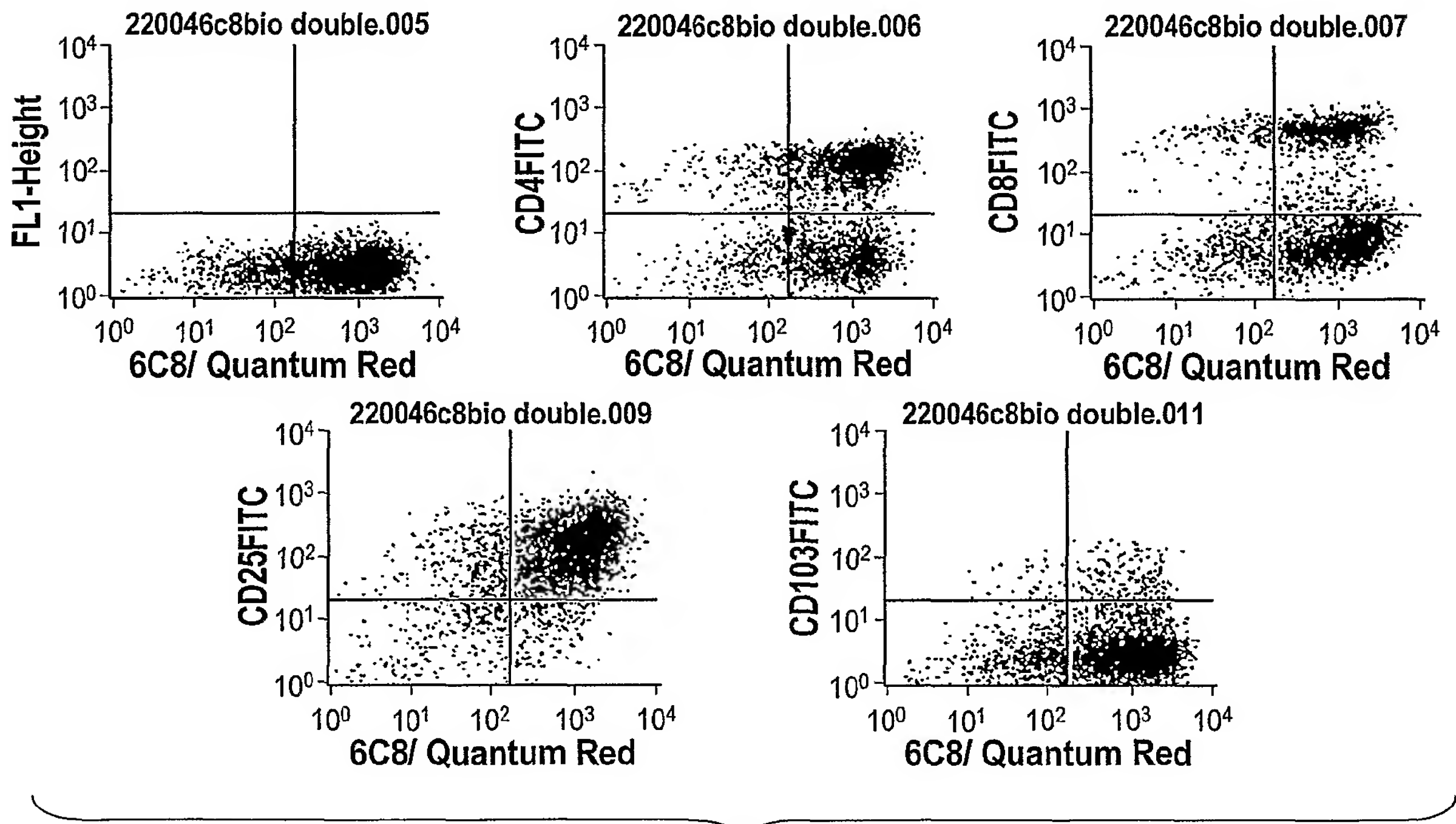


Fig. 4

3/13

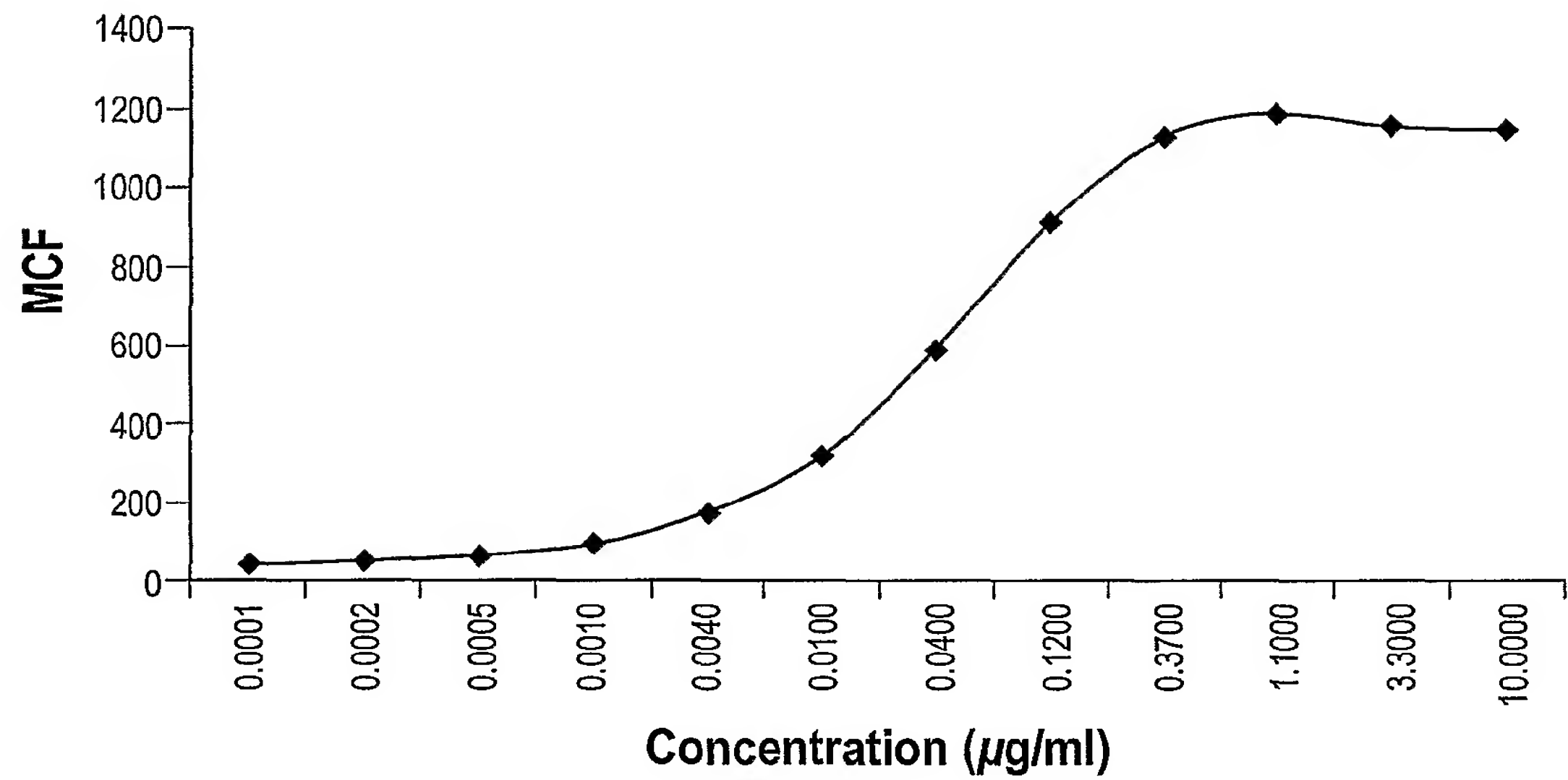


Fig. 5

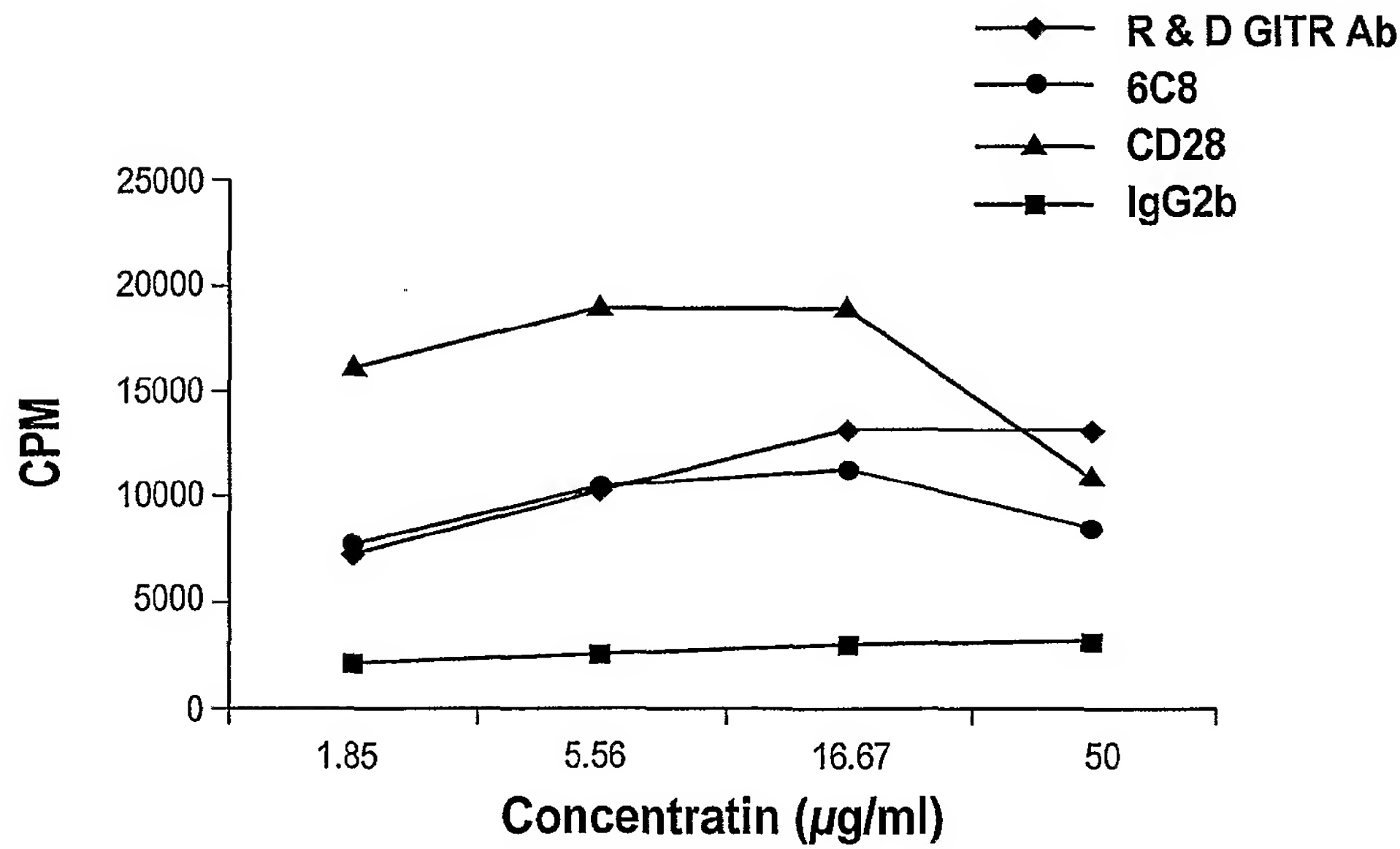


Fig. 6

4/13

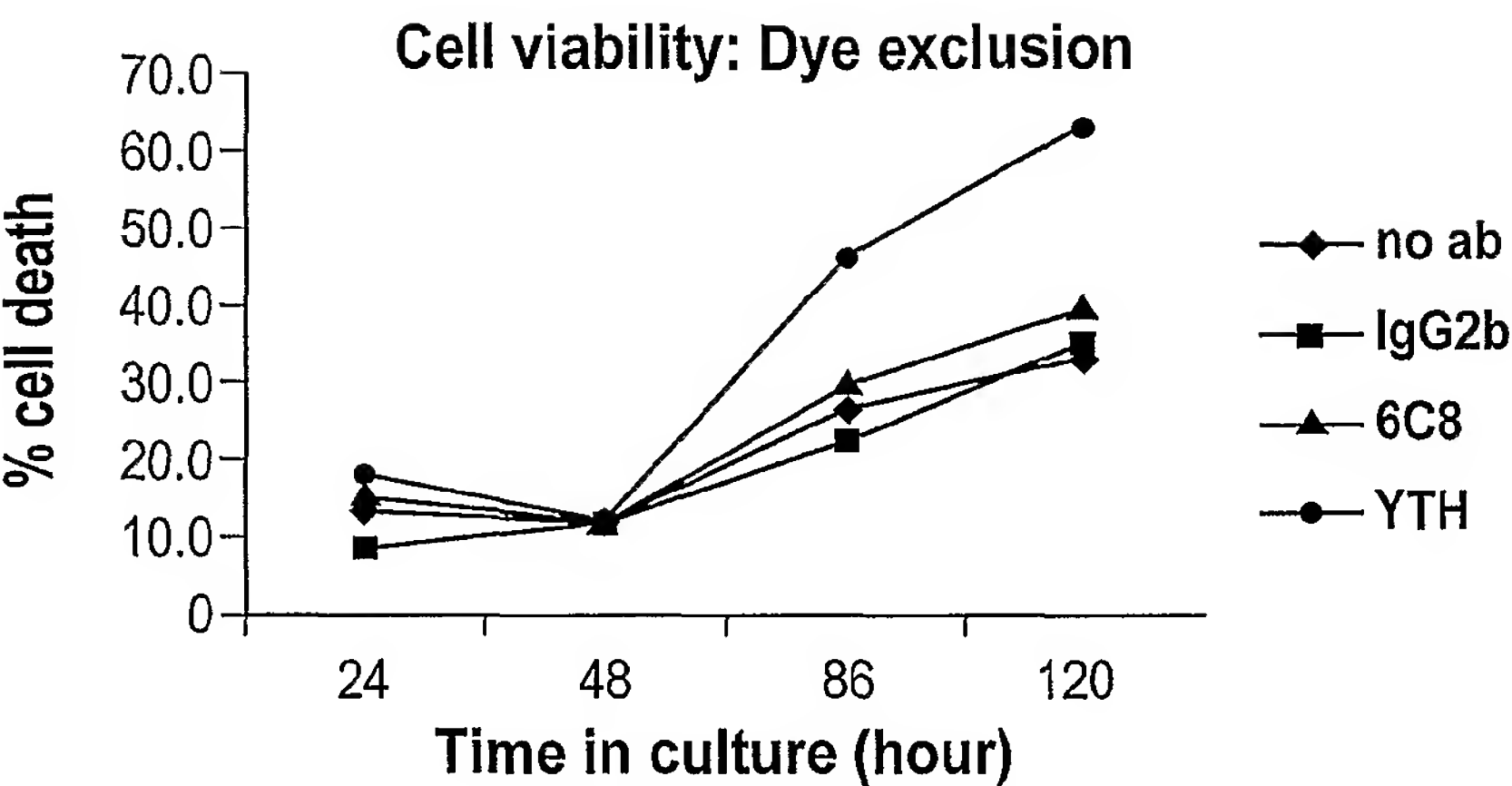


Fig. 7A

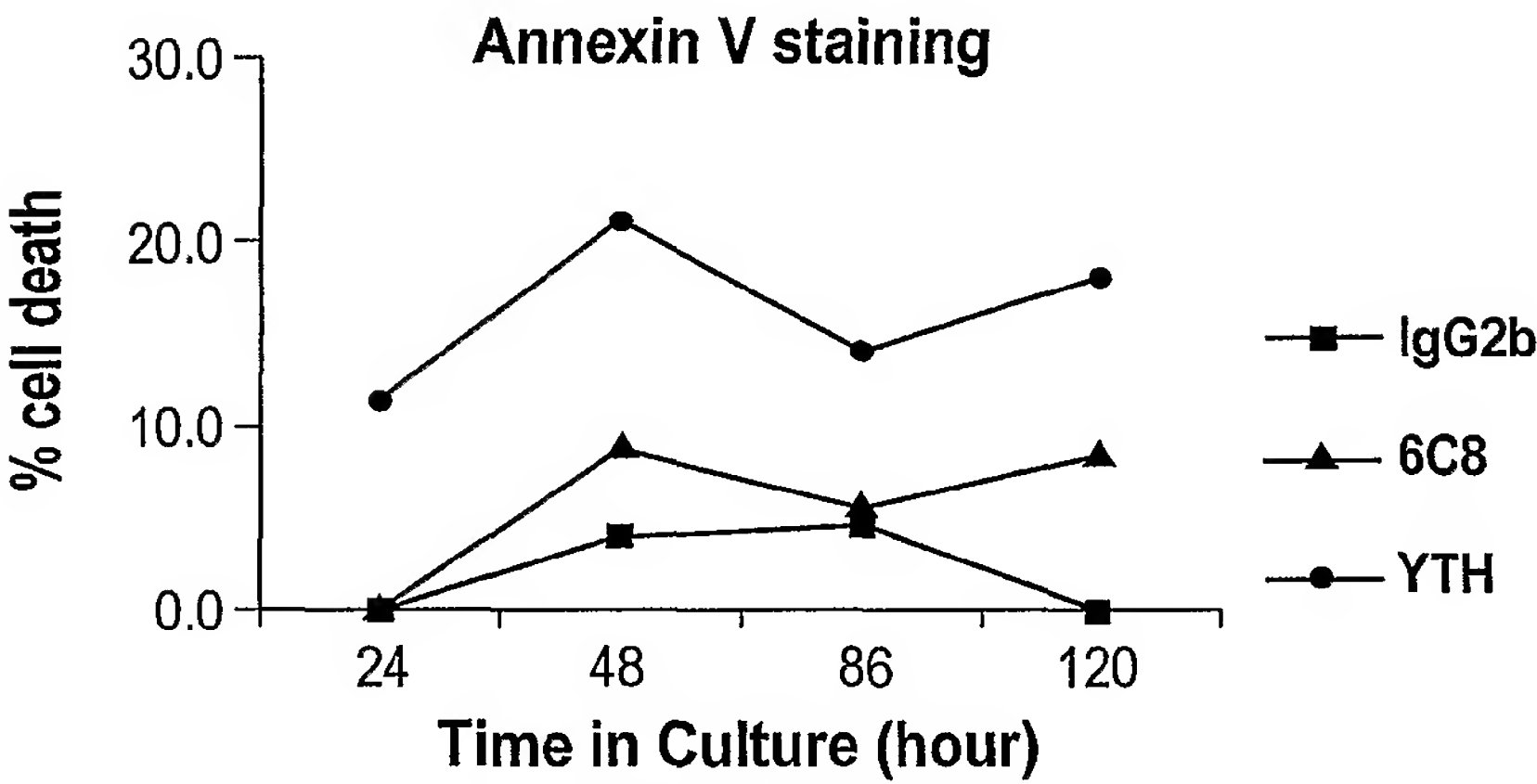


Fig. 7B

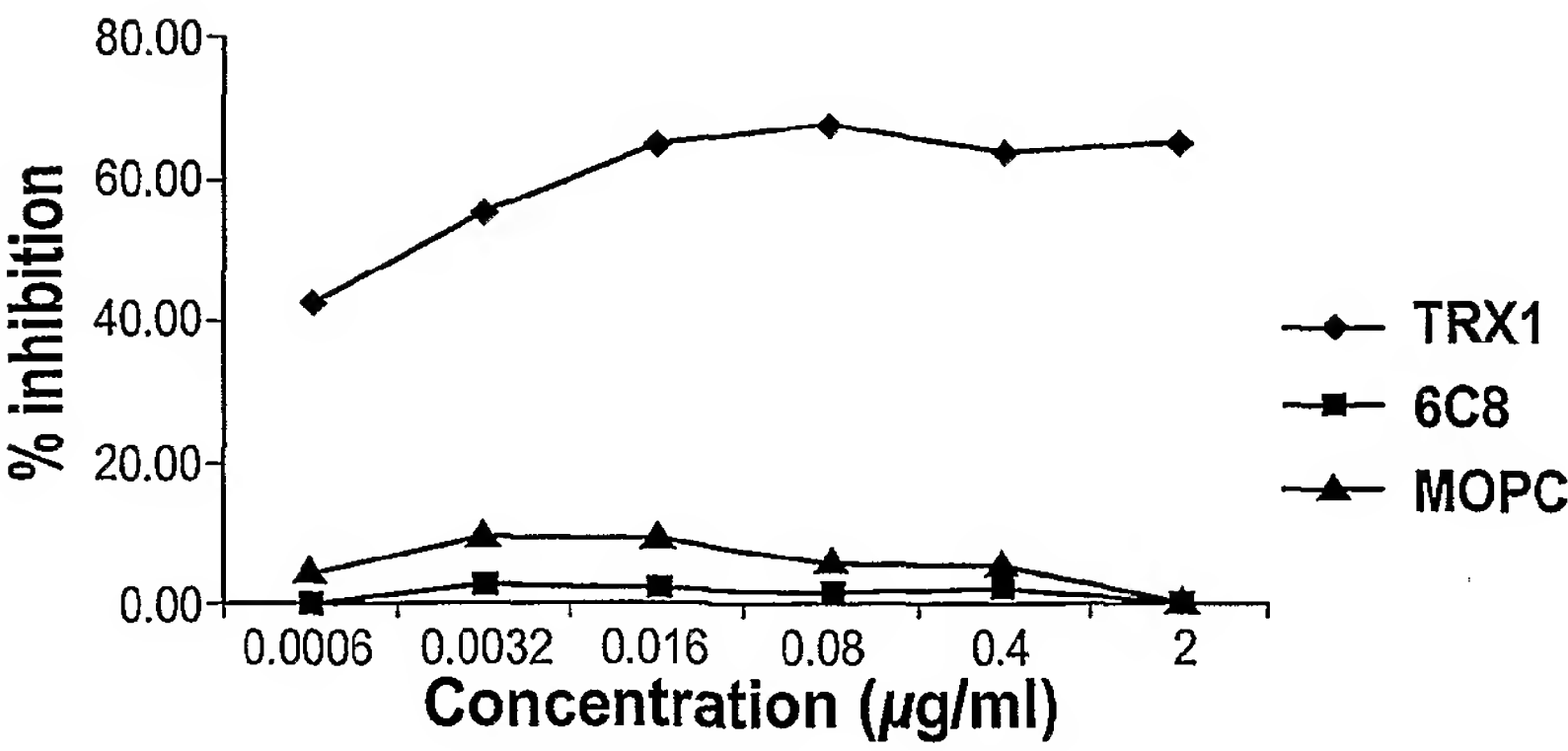
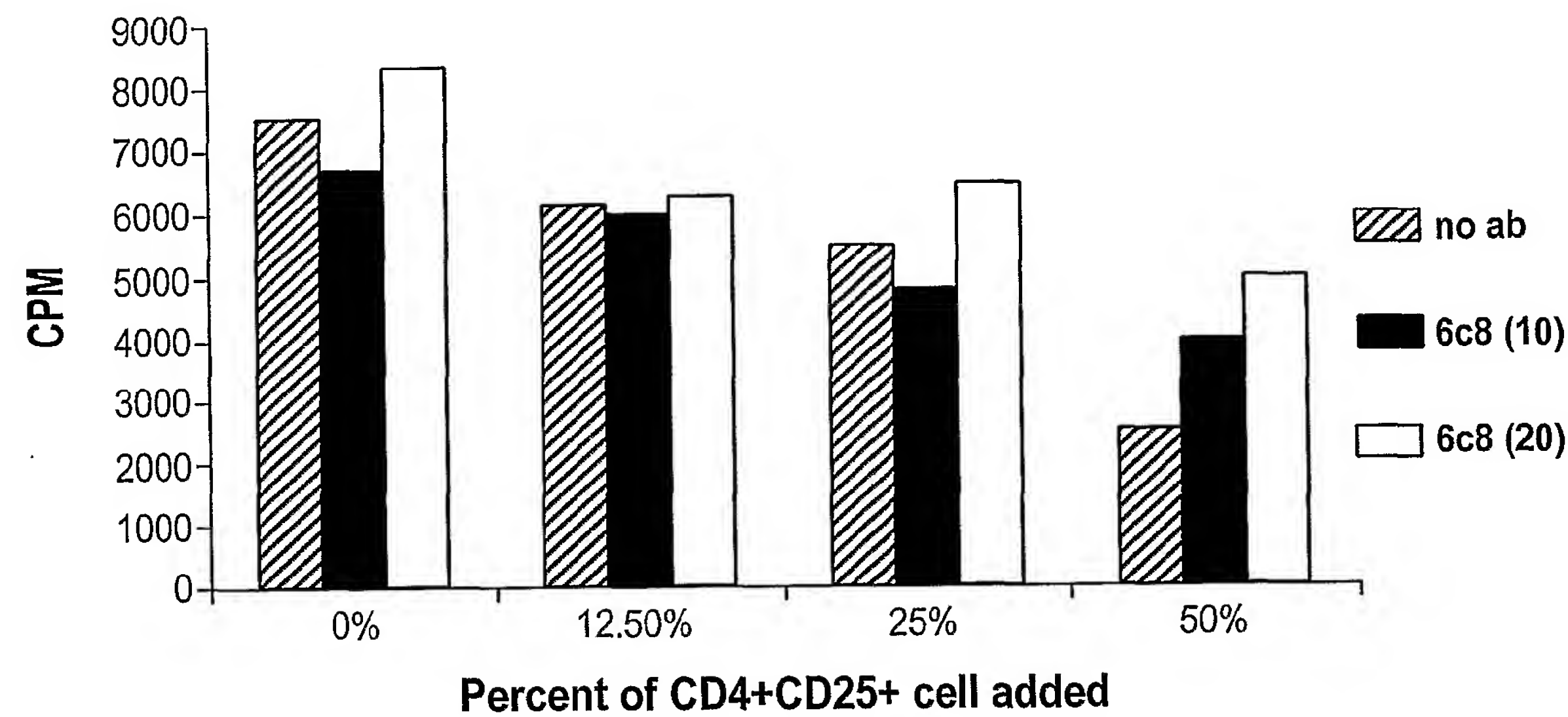
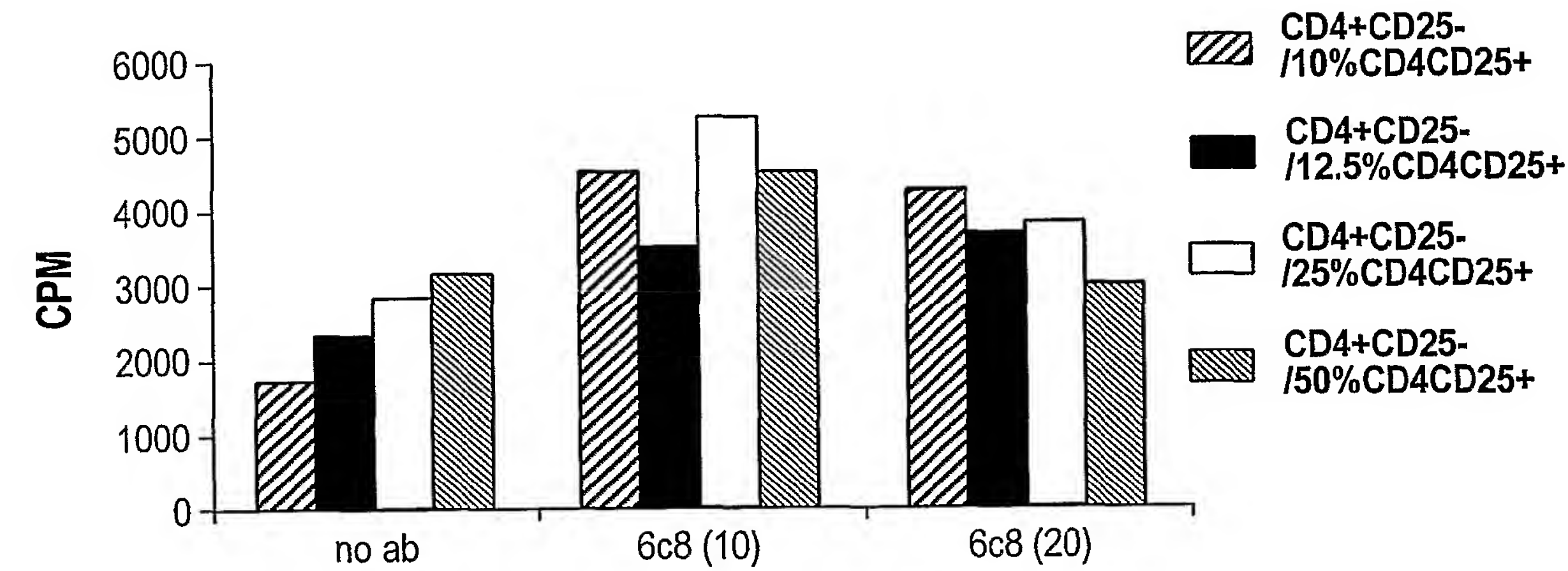


Fig. 8

5/13

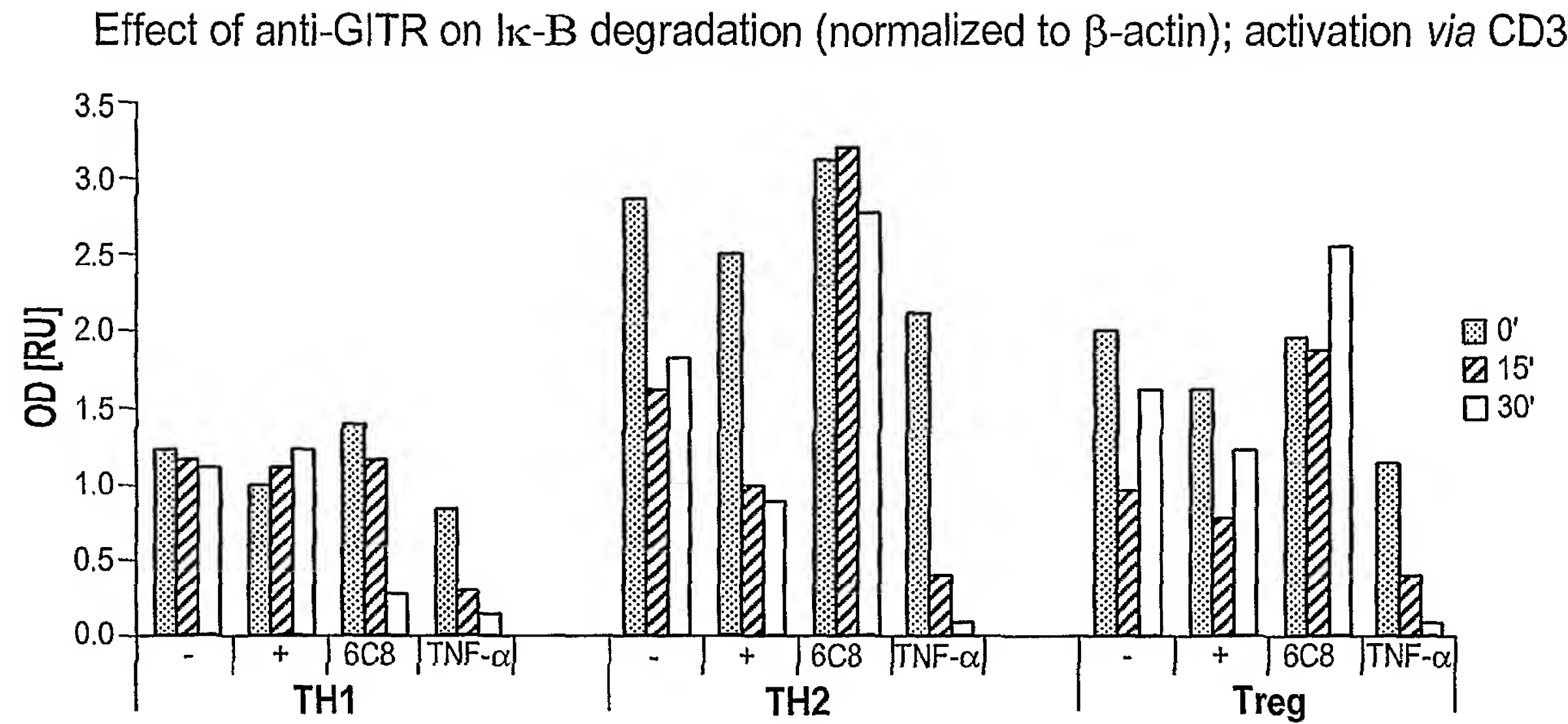


*Fig. 9*

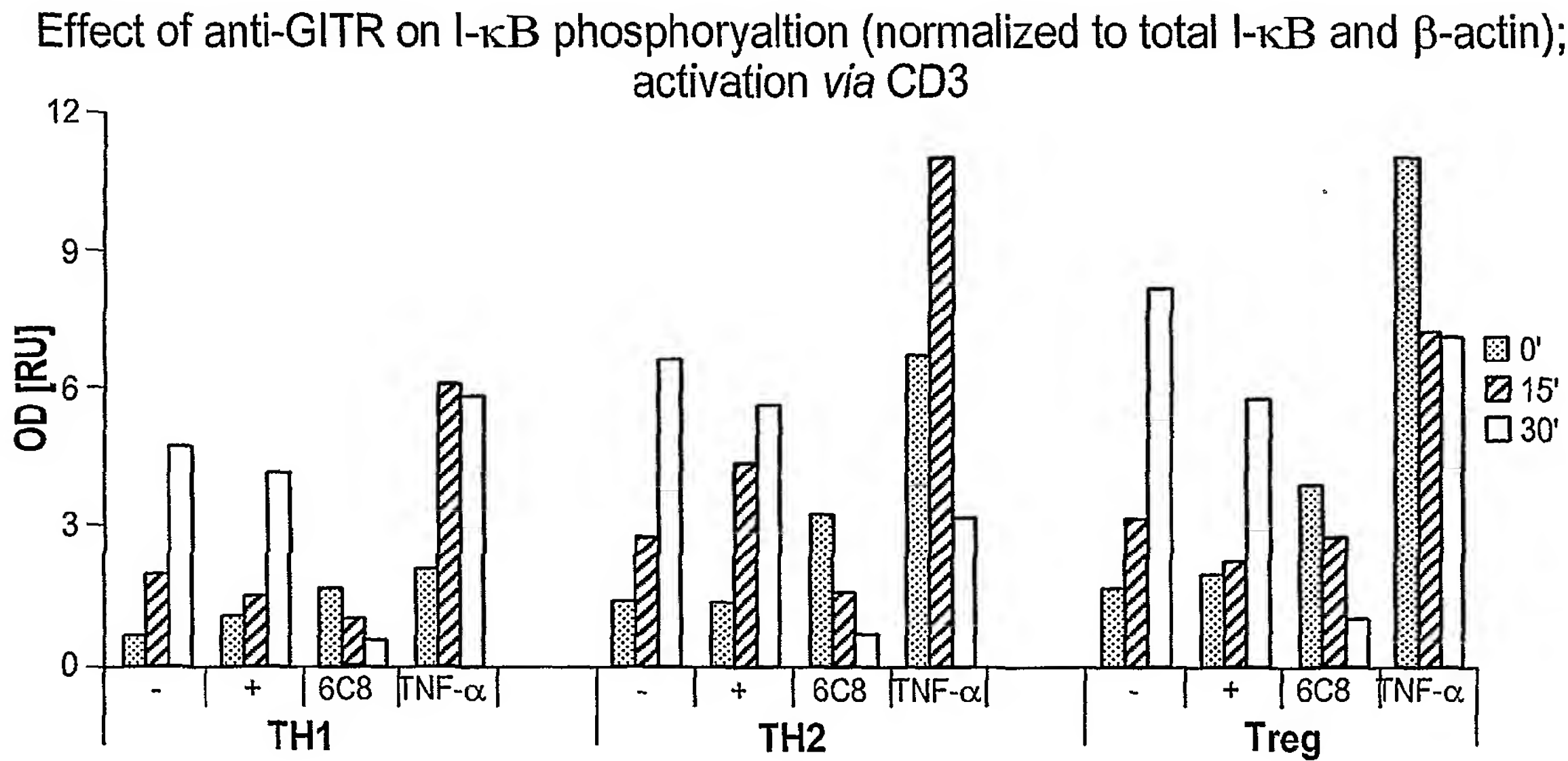


*Fig. 10*



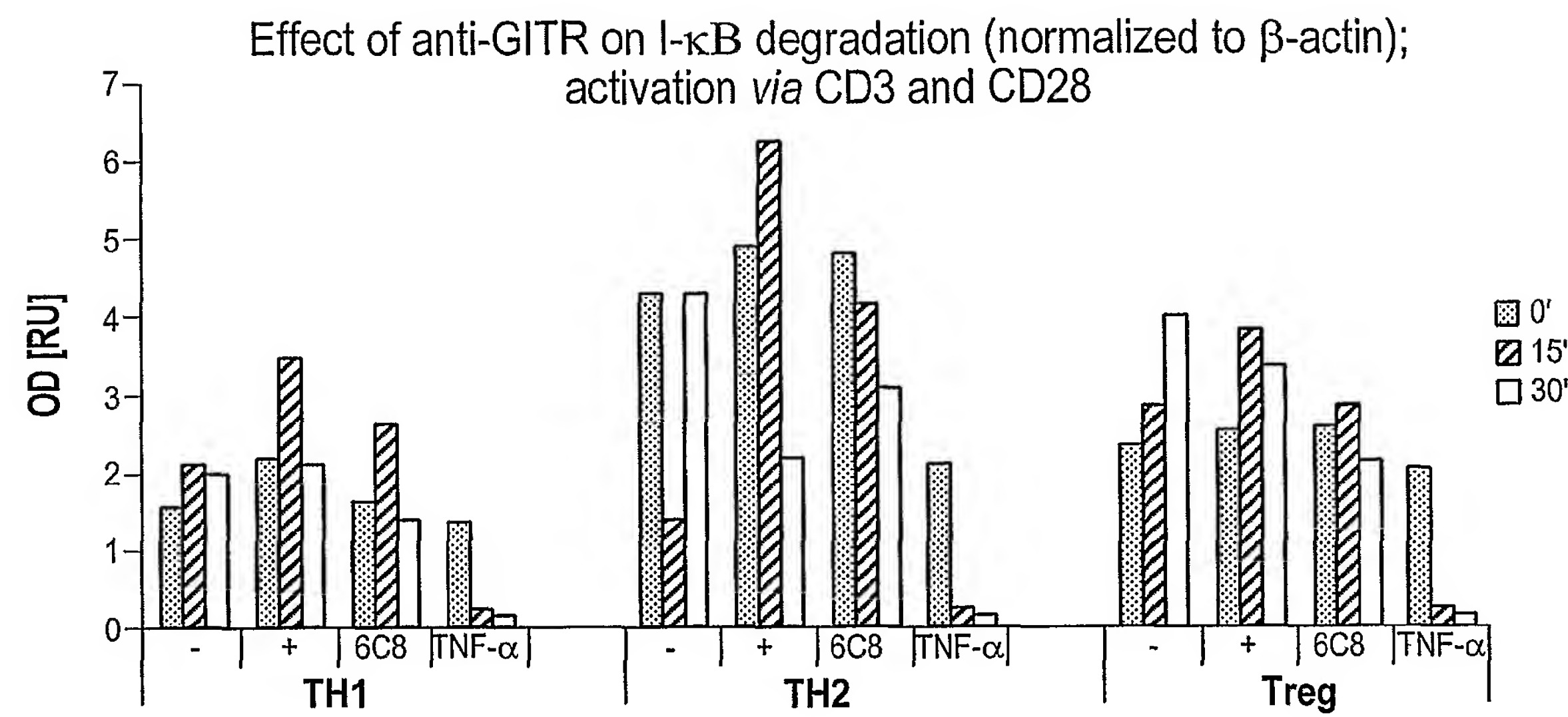


*Fig. 11*

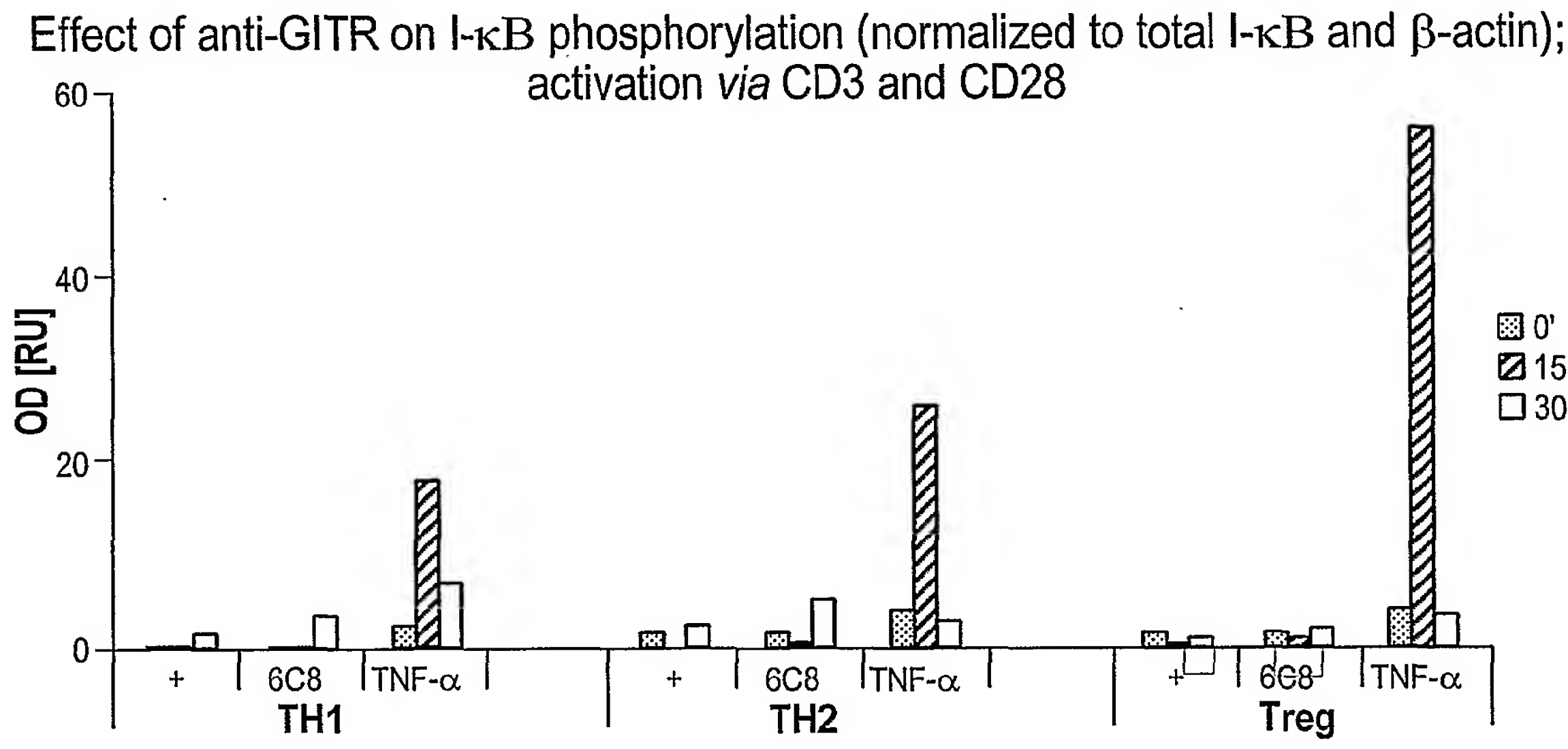


*Fig. 12*

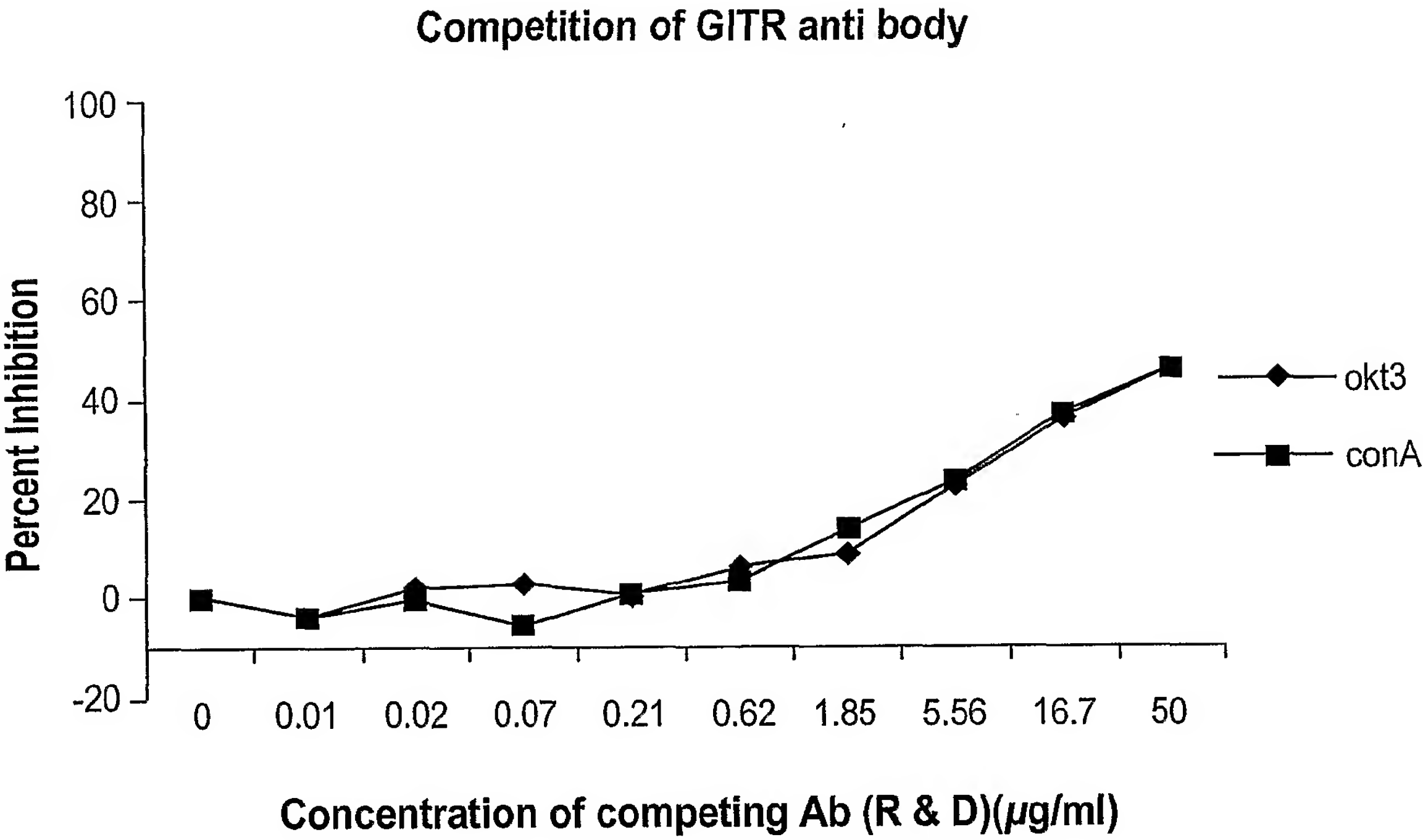
7/13



*Fig. 13*



*Fig. 14*



*Fig. 15*

Kinetic Analysis of G1TR Antibody

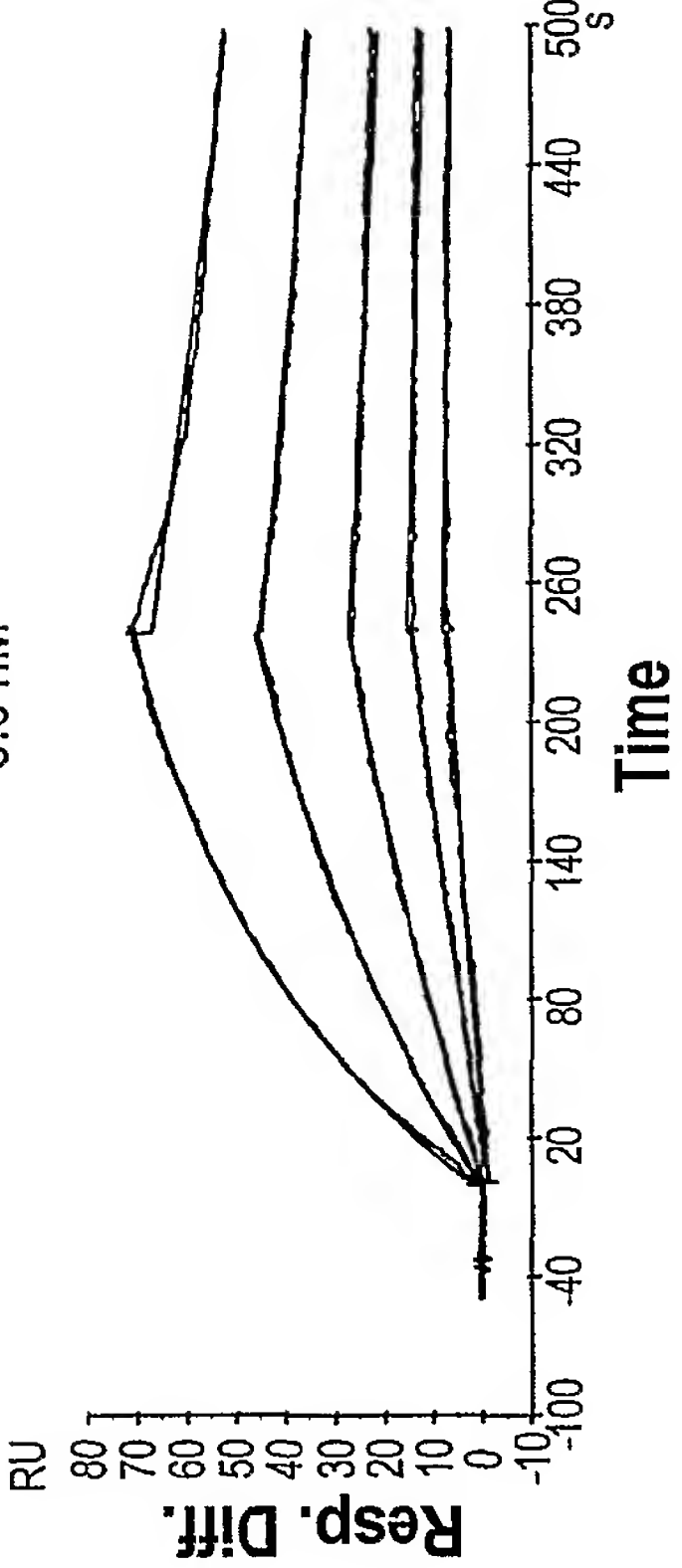
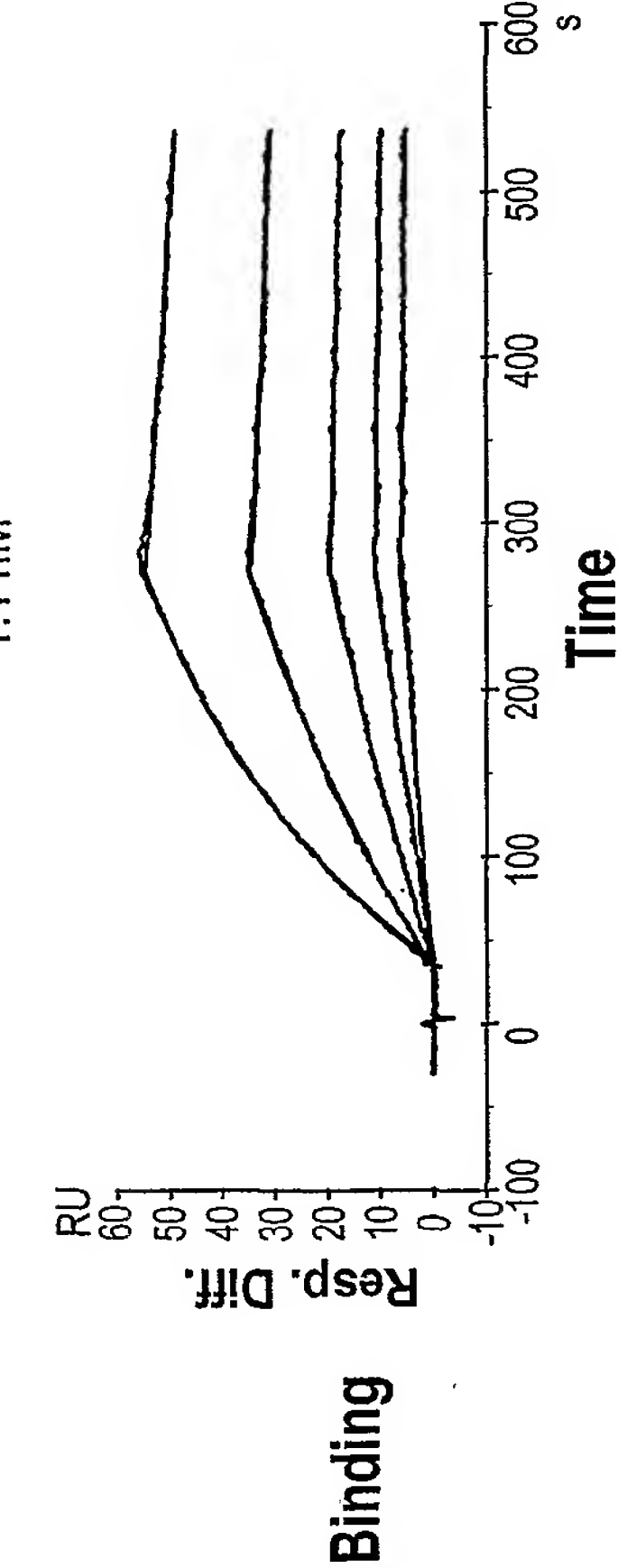
6C8

R&D

Affinity

1.1 nM

9.6 nM



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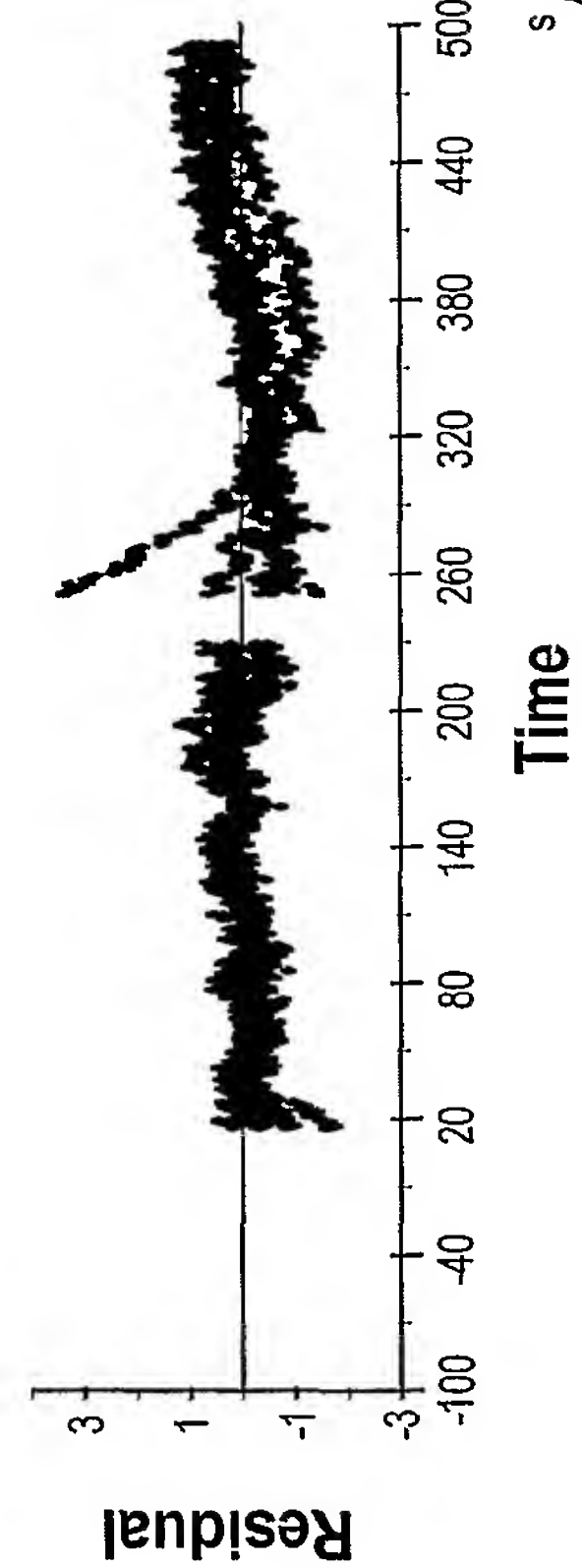
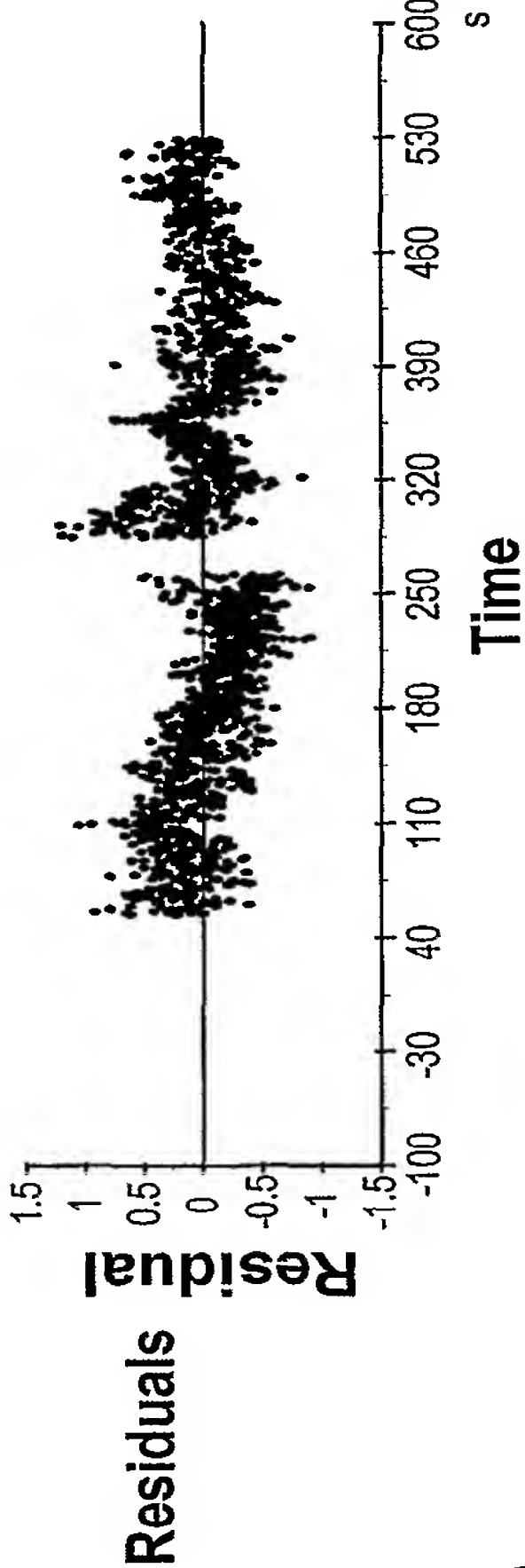


Fig. 16



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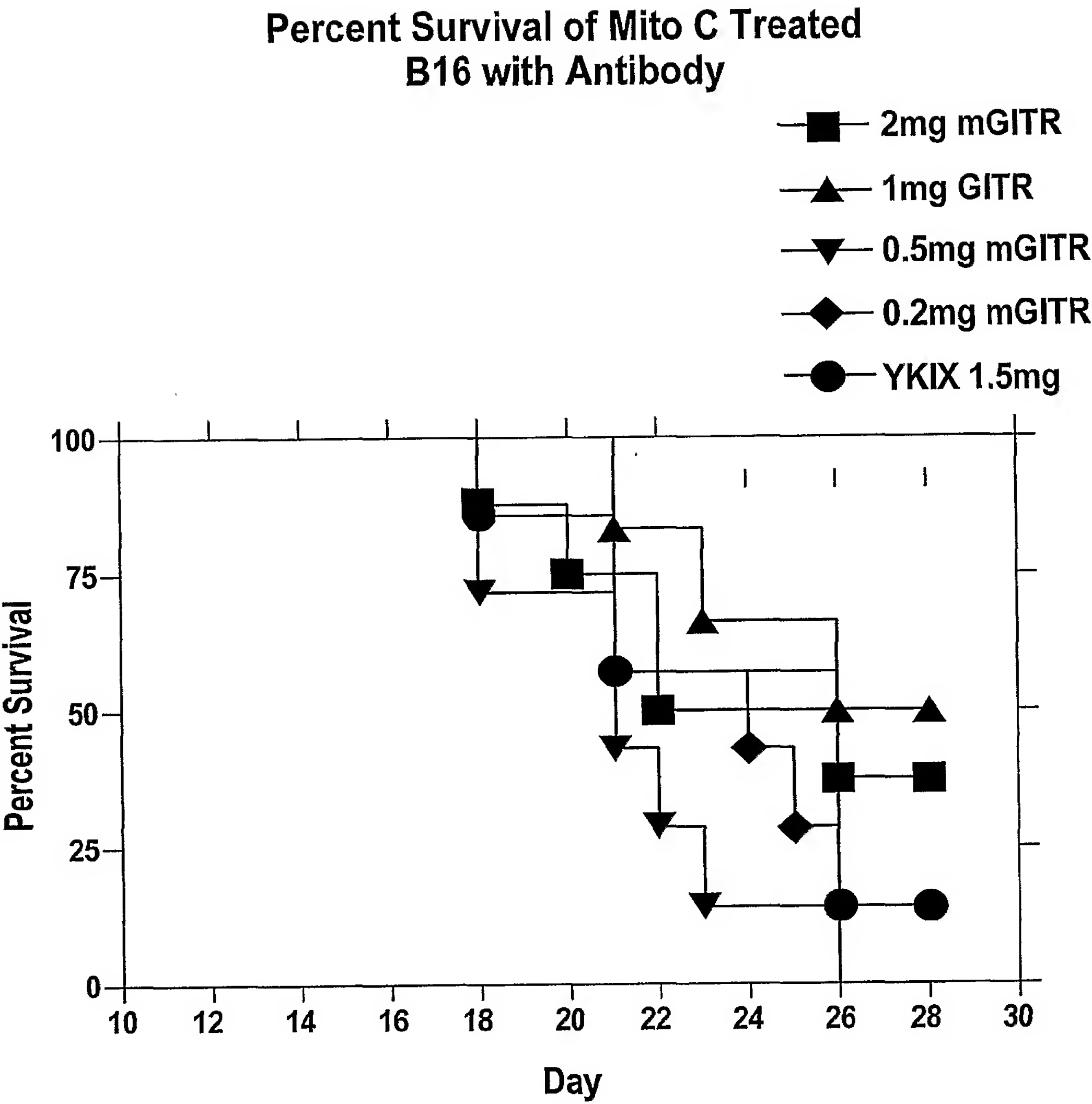


Fig. 17

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## 6C8 VHD

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B.

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## 6C8 VKA

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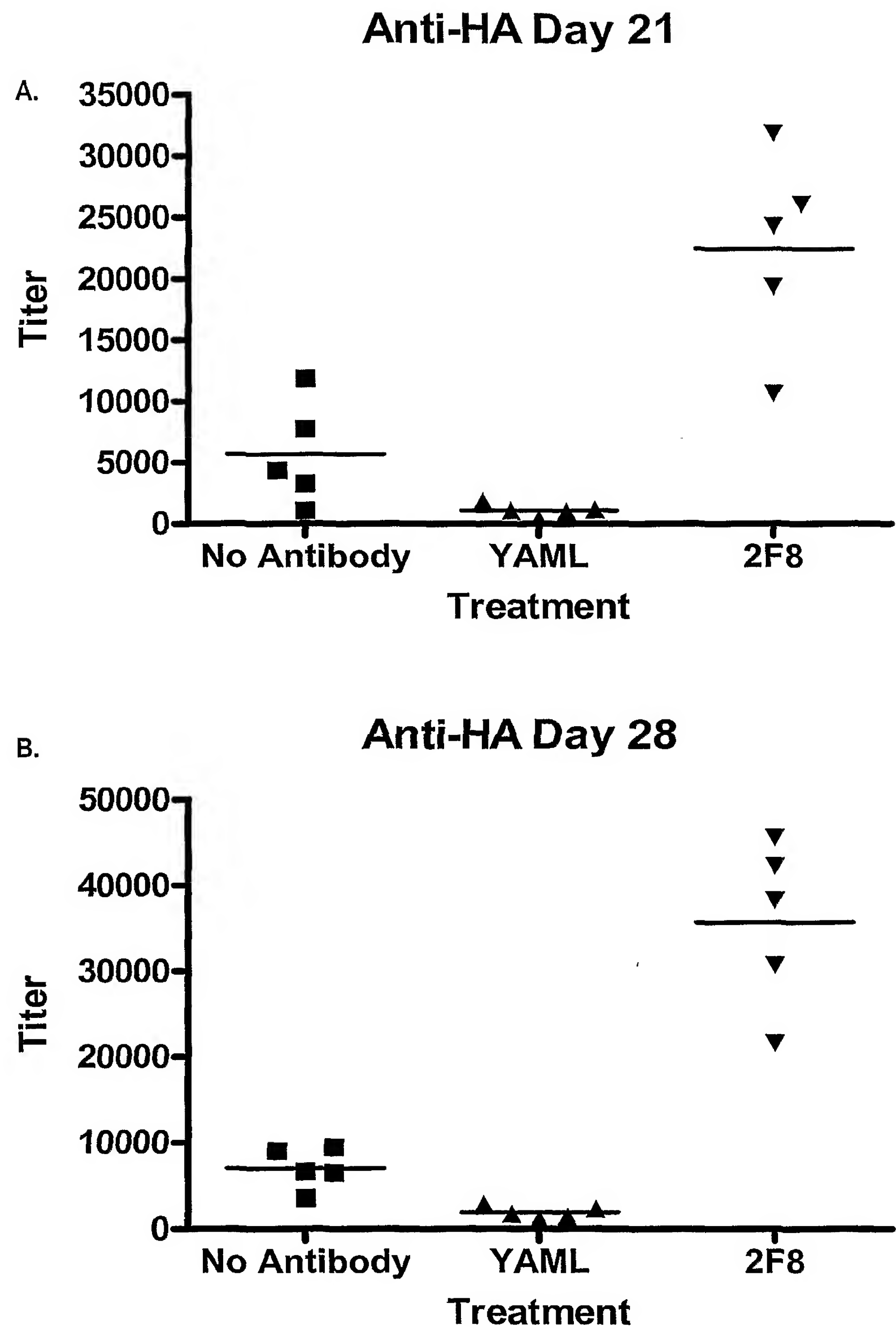
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D.

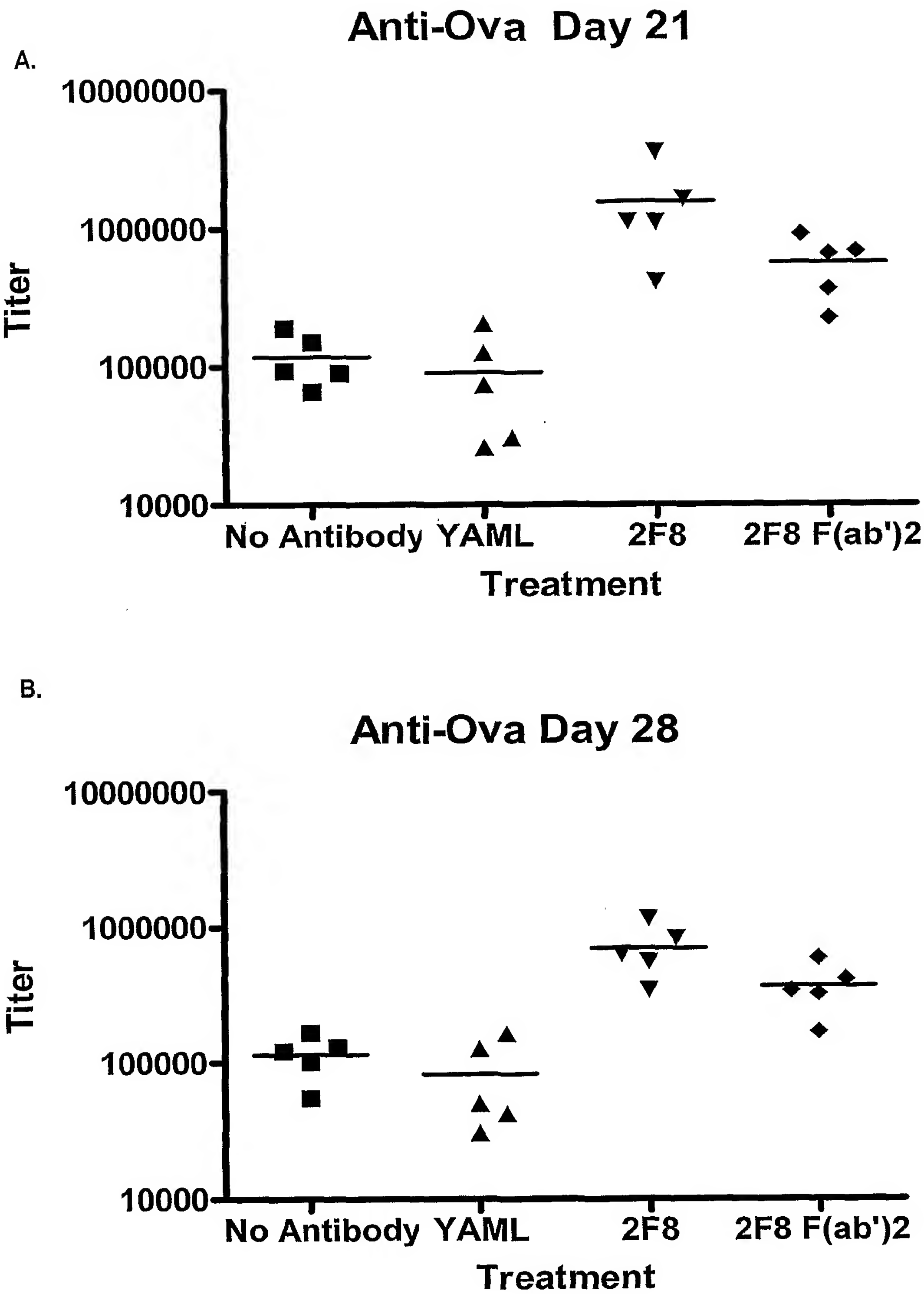
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*Fig. 18*

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*Fig. 19*



*Fig. 20*



## SEQUENCE LISTING

<110> SMITH, L. MARY  
 SZYMANSKA, GRAZYNA  
 PONATH, PAUL  
 ROSENZWEIG, MICHAEL

<120> GITR BINDING MOLECULES AND USES THEREFOR

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<151> 2005-03-25

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<400> 11
gggttttcac tgagcacttc tggatatgggt gtaggc 36

```

<210> 12  
 <211> 48  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 oligonucleotide

```

<400> 12
cacatttggt gggatgatga taagtactat aatccatccc tgaagagc 48

```

<210> 13  
 <211> 27  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 oligonucleotide

```

<400> 13
actaggaggt acttcccctt tgcttac 27

```

<210> 14



<211> 33  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 oligonucleotide

<400> 14  
 aaggccagtc agaatgtggg tactaatgta gcc 33

<210> 15  
 <211> 21  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 oligonucleotide

<400> 15  
 tcggcatcct accggtacag t 21

<210> 16  
 <211> 27  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 oligonucleotide

<400> 16  
 caacaatata acaccgatcc gctcacg 27

<210> 17  
 <211> 1214

<212> DNA  
 <213> Homo sapiens

<400> 17  
 gtctacaccc cctcctcaca cgcacttcac ctgggtcggg attctcaggt catgaacggt 60  
 cccagccacc tccgggcagg gcgggtgagg acggggacgg ggcgtgtcca actggctgtg 120  
 ggctcttgaa acccgagcat ggcacagcac ggggcgatgg gcgcgtttcg ggccctgtgc 180  
 ggccctggcg tgetgtgcgc gctcagcctg ggtcagcgcc ccaccggggg tcccgggtgc 240  
 ggccctgggc gcctcctgct tgggacggga acggacgcgc gctgctgccg gggtcacacg 300  
 acgcgctgct gccgcgatta cccgggcgag gagtgtgtt ccgagtggga ctgcatgtgt 360  
 gtccagcctg aattccactg cggagaccct tgetgcacga cctgccggca ccacccttgt 420  
 cccccaggcc agggggtaca gtcccagggg aaattcagtt ttggcttcca gtgtatcgac 480  
 tgtgcctcgg ggaccttctc cggggggccac gaaggccact gcaaaccctg gacagactgc 540  
 acccagttcg ggtttctcac tgtgttccct gggaacaaga ccacacacgc tgtgtgcgtc 600  
 ccagggtccc cgccggcaga gccgcttggg tggctgaccg tcgtcctcct ggccgtggcc 660  
 gcctgcgtcc tctccttgac ctccggcccag cttggactgc acatctggca gctgaggagt 720  
 cagtgcattg ggccccgaga gaccagctg ctgctggagg tgccgcgcgc gaccgaagac 780  
 gccagaagct gccagttccc cgaggaagag cggggcgagc gatcggcaga ggagaagggg 840  
 cggctgggag acctgtgggt gtgagcctgg ccgtcctccg gggccaccga ccgcagccag 900

```

cccccccca ggagctcccc aggcgcgagg ggctctgcgt tctgctctgg gccgggccc 960
gctccccctgg cagcagaagt ggggtgcagga aggtggcagt gaccagcgcc ctggaccatg 1020
cagttcggcg gccgcggctg ggccctgcag gagggagaga gagacacagt catggccccc 1080
ttctccctt gctggccctg atgggggtggg gtcttaggac gggaggctgt gtccgtgggt 1140
gtgcagtgcc cagcacggga cccggctgca ggggaccttc aataaacact tgtccagtga 1200
aaaaaaaaa aaaa 1214

```

&lt;210&gt; 18

&lt;211&gt; 241

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 18

```

Met Ala Gln His Gly Ala Met Gly Ala Phe Arg Ala Leu Cys Gly Leu
  1           5           10           15

```

```

Ala Leu Leu Cys Ala Leu Ser Leu Gly Gln Arg Pro Thr Gly Gly Pro
          20           25           30

```

```

Gly Cys Gly Pro Gly Arg Leu Leu Leu Gly Thr Gly Thr Asp Ala Arg
          35           40           45

```

```

Cys Cys Arg Val His Thr Thr Arg Cys Cys Arg Asp Tyr Pro Gly Glu
          50           55           60

```

```

Glu Cys Cys Ser Glu Trp Asp Cys Met Cys Val Gln Pro Glu Phe His
          65           70           75           80

```

```

Cys Gly Asp Pro Cys Cys Thr Thr Cys Arg His His Pro Cys Pro Pro
          85           90           95

```

```

Gly Gln Gly Val Gln Ser Gln Gly Lys Phe Ser Phe Gly Phe Gln Cys
          100          105          110

```

```

Ile Asp Cys Ala Ser Gly Thr Phe Ser Gly Gly His Glu Gly His Cys
          115          120          125

```

```

Lys Pro Trp Thr Asp Cys Thr Gln Phe Gly Phe Leu Thr Val Phe Pro
          130          135          140

```

```

Gly Asn Lys Thr His Asn Ala Val Cys Val Pro Gly Ser Pro Pro Ala
          145          150          155          160

```

```

Glu Pro Leu Gly Trp Leu Thr Val Val Leu Leu Ala Val Ala Ala Cys
          165          170          175

```

```

Val Leu Leu Leu Thr Ser Ala Gln Leu Gly Leu His Ile Trp Gln Leu
          180          185          190

```

```

Arg Ser Gln Cys Met Trp Pro Arg Glu Thr Gln Leu Leu Leu Glu Val
          195          200          205

```

```

Pro Pro Ser Thr Glu Asp Ala Arg Ser Cys Gln Phe Pro Glu Glu Glu
          210          215          220

```

```

Arg Gly Glu Arg Ser Ala Glu Glu Lys Gly Arg Leu Gly Asp Leu Trp
          225          230          235          240

```

Val

<210> 19  
 <211> 16  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic peptide

<400> 19  
 His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr Gln Pro Ser Leu Lys Ser  
           1                  5                  10                  15

<210> 20  
 <211> 105  
 <212> PRT  
 <213> Mus musculus

<400> 20  
 Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln  
           1                  5                  10                  15  
 Leu Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr  
                   20                  25                  30  
 Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln  
                   35                  40                  45  
 Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr  
           50                  55                  60  
 Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg  
           65                  70                  75                  80  
 His Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro  
                   85                  90                  95  
 Ile Val Lys Ser Phe Asn Arg Asn Glu  
           100                  105

<210> 21  
 <211> 334  
 <212> PRT  
 <213> Mus musculus

<400> 21  
 Ala Lys Thr Thr Pro Pro Ser Val Tyr Pro Leu Ala Pro Gly Cys Gly  
           1                  5                  10                  15  
 Asp Thr Thr Gly Ser Ser Val Thr Leu Gly Cys Leu Val Lys Gly Tyr  
                   20                  25                  30  
 Phe Pro Glu Ser Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser  
           35                  40                  45  
 Ser Val His Thr Phe Pro Ala Leu Leu Gln Ser Gly Leu Tyr Thr Met

50					55					60					
Ser 65	Ser	Ser	Val	Thr	Val 70	Pro	Ser	Ser	Thr	Trp 75	Pro	Ser	Gln	Thr	Val 80
Thr	Cys	Ser	Val	Ala 85	His	Pro	Ala	Ser	Ser 90	Thr	Thr	Val	Asp	Lys 95	Lys
Leu	Glu	Pro	Ser 100	Gly	Pro	Ile	Ser	Thr 105	Ile	Asn	Pro	Cys	Pro 110	Pro	Cys
Lys	Glu	Cys 115	Lys	Cys	Pro	Ala	Pro 120	Asn	Leu	Glu	Gly 125	Gly	Pro	Ser	Val
Phe 130	Ile	Phe	Pro	Pro	Asn	Ile 135	Lys	Asp	Val	Leu	Met 140	Ile	Ser	Leu	Thr
Pro 145	Lys	Val	Thr	Cys	Val 150	Val	Val	Asp	Val 155	Ser	Glu	Asp	Asp	Pro	Asp 160
Val	Gln	Ile	Ser	Trp 165	Phe	Val	Asn	Asn	Val 170	Glu	Val	His	Thr	Ala	Gln 175
Thr	Gln	Thr	His 180	Arg	Glu	Asp	Tyr	Asn 185	Ser	Thr	Ile	Arg	Val 190	Val	Ser
Thr	Leu	Pro 195	Ile	Gln	His	Gln	Asp 200	Trp	Met	Ser	Gly	Lys 205	Glu	Phe	Lys
Cys 210	Lys	Val	Asn	Asn	Lys	Asp 215	Leu	Pro	Ser	Pro	Ile 220	Glu	Arg	Thr	Ile
Ser 225	Lys	Ile	Lys	Gly	Leu 230	Val	Arg	Ala	Gln	Val 235	Tyr	Ile	Leu	Pro	Pro 240
Pro	Ala	Glu	Gln	Leu 245	Ser	Arg	Lys	Asp	Val 250	Ser	Leu	Thr	Cys	Leu	Val 255
Val	Gly	Phe	Asn 260	Pro	Gly	Asp	Ile	Ser 265	Val	Glu	Trp	Thr	Ser	Asn	Gly
His	Thr	Glu	Glu	Asn	Tyr	Lys	Asp 280	Thr	Ala	Pro	Val	Leu	Asp 285	Ser	Asp
Gly 290	Ser	Tyr	Phe	Ile	Tyr	Ser 295	Lys	Leu	Asn	Met	Lys 300	Thr	Ser	Lys	Trp
Glu 305	Lys	Thr	Asp	Ser	Phe	Ser 310	Cys	Asn	Val	Arg 315	His	Glu	Gly	Leu	Lys 320
Asn	Tyr	Tyr	Leu	Lys 325	Lys	Thr	Ile	Ser	Arg 330	Ser	Pro	Gly	Lys		

&lt;210&gt; 22

&lt;211&gt; 214

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Synthetic



mouse/human light chain construct

<400> 22

Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly  
1 5 10 15

Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn  
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile  
35 40 45

Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Asn Asn Val His Ser  
65 70 75 80

Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Thr Asp Pro Leu  
85 90 95

Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Arg Thr Val Ala Ala  
100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
195 200 205

Phe Asn Arg Gly Glu Cys  
210

<210> 23

<211> 449

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
mouse/human heavy chain construct

<400> 23

Gln Val Thr Leu Lys Glu Ser Gly Pro Gly Ile Leu Lys Pro Ser Gln  
1 5 10 15

Thr Leu Ser Leu Thr Cys Ser Phe Ser Gly Phe Ser Leu Ser Thr Ser

20					25					30					
Gly	Met	Gly	Val	Gly	Trp	Ile	Arg	Gln	Pro	Ser	Gly	Lys	Gly	Leu	Glu
		35					40					45			
Trp	Leu	Ala	His	Ile	Trp	Trp	Asp	Asp	Asp	Lys	Tyr	Tyr	Asn	Pro	Ser
	50					55					60				
Leu	Lys	Ser	Gln	Leu	Thr	Ile	Ser	Lys	Asp	Thr	Ser	Arg	Asn	Gln	Val
	65				70					75					80
Phe	Leu	Lys	Ile	Thr	Ser	Val	Asp	Thr	Ala	Asp	Ala	Ala	Thr	Tyr	Tyr
				85					90					95	
Cys	Ala	Arg	Thr	Arg	Arg	Tyr	Phe	Pro	Phe	Ala	Tyr	Trp	Gly	Gln	Gly
			100					105					110		
Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe
		115					120					125			
Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu
	130					135					140				
Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp
145					150					155					160
Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu
				165					170					175	
Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
			180					185					190		
Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro
		195					200					205			
Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys
	210					215					220				
Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
225					230					235					240
Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser
				245					250					255	
Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp
			260					265					270		
Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn
		275					280					285			
Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val
	290					295					300				
Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu
305					310					315					320
Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys
				325					330					335	
Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr
			340					345					350		

Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
 355 360 365  
 Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
 370 375 380  
 Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
 385 390 395 400  
 Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
 405 410 415  
 Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
 420 425 430  
 Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
 435 440 445

Lys

<210> 24  
 <211> 449  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 mouse/human complete chimeric heavy chain

<400> 24  
 Gln Val Thr Leu Lys Glu Ser Gly Pro Gly Ile Leu Lys Pro Ser Gln  
 1 5 10 15  
 Thr Leu Ser Leu Thr Cys Ser Phe Ser Gly Phe Ser Leu Ser Thr Ser  
 20 25 30  
 Gly Met Gly Val Gly Trp Ile Arg Gln Pro Ser Gly Lys Gly Leu Glu  
 35 40 45  
 Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr Asn Pro Ser  
 50 55 60  
 Leu Lys Ser Gln Leu Thr Ile Ser Lys Asp Thr Ser Arg Asn Gln Val  
 65 70 75 80  
 Phe Leu Lys Ile Thr Ser Val Asp Thr Ala Asp Ala Ala Thr Tyr Tyr  
 85 90 95  
 Cys Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Gln Gly  
 100 105 110  
 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125  
 Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
 130 135 140  
 Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp

145					150					155					160
Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu
				165					170					175	
Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
			180					185					190		
Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro
		195					200					205			
Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys
	210					215					220				
Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
225					230					235					240
Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser
				245					250					255	
Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp
			260					265					270		
Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn
		275					280					285			
Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Ala	Ser	Thr	Tyr	Arg	Val
	290					295					300				
Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu
305					310					315					320
Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys
				325					330					335	
Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr
			340					345					350		
Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr
		355					360					365			
Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu
	370					375					380				
Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu
385					390					395					400
Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys
				405					410					415	
Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu
			420					425					430		
Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly
		435					440					445			
Lys															



&lt;210&gt; 25

&lt;211&gt; 95

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 25

Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly  
1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Asn  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
35 40 45

Tyr Gly Ala Ser Thr Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser  
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Asn Asn Trp Pro  
85 90 95

&lt;210&gt; 26

&lt;211&gt; 95

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 26

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly  
1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Gly Val Ser Ser Tyr  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
35 40 45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
50 55 60

Ser Gly Pro Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp His  
85 90 95

&lt;210&gt; 27

&lt;211&gt; 95

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 27

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly  
1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Tyr

20 25 30  
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
 35 40 45  
 Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
 65 70 75 80  
 Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Pro  
 85 90 95

<210> 28  
 <211> 95  
 <212> PRT  
 <213> Homo sapiens

<400> 28  
 Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly  
 1 5 10 15  
 Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Tyr  
 20 25 30  
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
 35 40 45  
 Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Arg Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
 65 70 75 80  
 Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Pro  
 85 90 95

<210> 29  
 <211> 95  
 <212> PRT  
 <213> Homo sapiens

<400> 29  
 Ala Ile Arg Met Thr Gln Ser Pro Phe Ser Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Thr Cys Trp Ala Ser Gln Gly Ile Ser Ser Tyr  
 20 25 30  
 Leu Ala Trp Tyr Gln Gln Lys Pro Ala Lys Ala Pro Lys Leu Phe Ile  
 35 40 45  
 Tyr Tyr Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ser Thr Pro  
85 90 95

<210> 30  
<211> 95  
<212> PRT  
<213> Homo sapiens

<400> 30  
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
1 5 10 15  
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Tyr  
20 25 30  
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
35 40 45  
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60  
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80  
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ser Tyr Ser Thr Pro  
85 90 95

<210> 31  
<211> 95  
<212> PRT  
<213> Homo sapiens

<400> 31  
Asp Ile Gln Met Thr Gln Ser Pro Ser Phe Leu Ser Ala Ser Val Gly  
1 5 10 15  
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Tyr  
20 25 30  
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
35 40 45  
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60  
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80  
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Cys Gly Tyr Ser Thr Pro  
85 90 95

<210> 32  
<211> 95  
<212> PRT  
<213> Homo sapiens

<400> 32  
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly

```
<210> 33
<211> 95
<212> PRT
<213> Homo sapiens
```

```

<400> 33
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
  1                      5                      10                      15
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Asn Tyr
          20                      25                      30
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Val Pro Lys Leu Leu Ile
          35                      40                      45
Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
          50                      55                      60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
  65                      70                      75                      80
Glu Asp Val Ala Thr Tyr Tyr Cys Gln Lys Tyr Asn Ser Ala Pro
          85                      90                      95

```

```
<210> 34
<211> 95
<212> PRT
<213> Homo sapiens
```

```

<400> 34
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
  1             5             10             15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Asp
          20             25             30

Leu Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Arg Leu Ile
          35             40             45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
  50             55             60

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Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Asn Ser Tyr Pro  
85 90 95

<210> 35  
<211> 95  
<212> PRT  
<213> Homo sapiens

<400> 35  
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
1 5 10 15  
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Asp  
20 25 30  
Leu Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Arg Leu Ile  
35 40 45  
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60  
Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Asn Leu Gln Pro  
65 70 75 80  
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Asn Ser Tyr Pro  
85 90 95

<210> 36  
<211> 95  
<212> PRT  
<213> Homo sapiens

<400> 36  
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
1 5 10 15  
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Trp  
20 25 30  
Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile  
35 40 45  
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60  
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80  
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Pro  
85 90 95

<210> 37  
<211> 95  
<212> PRT  
<213> Homo sapiens

&lt;400&gt; 37

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Arg Gln Gly Ile Ser Ser Trp  
 20 25 30  
 Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile  
 35 40 45  
 Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Pro  
 85 90 95

&lt;210&gt; 38

&lt;211&gt; 95

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 38

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Asn Tyr  
 20 25 30  
 Leu Ala Trp Phe Gln Gln Lys Pro Gly Lys Ala Pro Lys Ser Leu Ile  
 35 40 45  
 Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Pro  
 85 90 95

&lt;210&gt; 39

&lt;211&gt; 95

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 39

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Val Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Trp  
 20 25 30  
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
 35 40 45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ala Asn Ser Phe Pro  
85 90 95

<210> 40

<211> 95

<212> PRT

<213> Homo sapiens

<400> 40

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Val Ser Ala Ser Val Gly  
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Trp  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
35 40 45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ala Asn Ser Phe Pro  
85 90 95

<210> 41

<211> 95

<212> PRT

<213> Homo sapiens

<400> 41

Asp Ile Gln Leu Thr Gln Ser Pro Ser Phe Leu Ser Ala Ser Val Gly  
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Tyr  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
35 40 45

Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Leu Asn Ser Tyr Pro  
85 90 95

<210> 42

<211> 95  
 <212> PRT  
 <213> Homo sapiens

<400> 42  
 Ala Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
     1                    5                    10                    15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Asp  
             20                    25                    30  
 Leu Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
             35                    40                    45  
 Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
             50                    55                    60  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
     65                    70                    75                    80  
 Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln Asp Tyr Asn Tyr Pro  
             85                    90                    95

<210> 43  
 <211> 95  
 <212> PRT  
 <213> Homo sapiens

<400> 43  
 Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Val Gly  
     1                    5                    10                    15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Trp  
             20                    25                    30  
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
             35                    40                    45  
 Tyr Asp Ala Ser Ser Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly  
             50                    55                    60  
 Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
     65                    70                    75                    80  
 Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Ser  
             85                    90                    95

<210> 44  
 <211> 107  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
         protein construct

<400> 44  
 Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly  
     1                    5                    10                    15



Glu Arg Ala Thr Leu Ser Cys Lys Ala Ser Gln Asn Val Gly Thr Asn  
                   20                  25                  30  
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
                   35                  40                  45  
 Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Ile Pro Ala Arg Phe Ser Gly  
           50                  55                  60  
 Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser  
   65                  70                  75                  80  
 Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Asn Thr Asp Pro Leu  
                   85                  90                  95  
 Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys  
                   100                  105

<210> 45  
 <211> 96  
 <212> PRT  
 <213> Homo sapiens

<400> 45  
 Gln Ile Thr Leu Lys Glu Ser Gly Pro Thr Leu Val Lys Pro Thr Gln  
   1                  5                  10                  15  
 Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
           20                  25                  30  
 Gly Val Gly Val Gly Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
           35                  40                  45  
 Trp Leu Ala Leu Ile Tyr Trp Asn Asp Asp Lys Arg Tyr Ser Pro Ser  
   50                  55                  60  
 Leu Lys Ser Arg Leu Thr Ile Thr Lys Asp Thr Ser Lys Asn Gln Val  
   65                  70                  75                  80  
 Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
           85                  90                  95

<210> 46  
 <211> 100  
 <212> PRT  
 <213> Homo sapiens

<400> 46  
 Gln Val Thr Leu Lys Glu Ser Gly Pro Val Leu Val Lys Pro Thr Glu  
   1                  5                  10                  15  
 Thr Leu Thr Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Ser Asn Ala  
           20                  25                  30  
 Arg Met Gly Val Ser Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu

35                                      40                                      45  
 Trp Leu Ala His Ile Phe Ser Asn Asp Glu Lys Ser Tyr Ser Thr Ser  
     50                                      55                                      60  
 Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Ser Gln Val  
     65                                      70                                      75                                      80  
 Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
                                     85                                      90                                      95  
 Cys Ala Arg Ile  
                                     100

<210> 47  
 <211> 100  
 <212> PRT  
 <213> Homo sapiens

<400> 47  
 Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
     1                                      5                                      10                                      15  
 Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
                                     20                                      25                                      30  
 Gly Met Cys Val Ser Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
                                     35                                      40                                      45  
 Trp Leu Ala Leu Ile Asp Trp Asp Asp Asp Lys Tyr Tyr Ser Thr Ser  
     50                                      55                                      60  
 Leu Lys Thr Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val  
     65                                      70                                      75                                      80  
 Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
                                     85                                      90                                      95  
 Cys Ala Arg Ile  
                                     100

<210> 48  
 <211> 99  
 <212> PRT  
 <213> Homo sapiens

<400> 48  
 Gln Leu Gln Leu Gln Glu Ser Gly Ser Gly Leu Val Lys Pro Ser Gln  
     1                                      5                                      10                                      15  
 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Gly Ser Ile Ser Ser Gly  
                                     20                                      25                                      30  
 Gly Tyr Ser Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu  
                                     35                                      40                                      45  
 Trp Ile Gly Tyr Ile Tyr His Ser Gly Ser Thr Tyr Tyr Asn Pro Ser  
     50                                      55                                      60

Leu Lys Ser Arg Val Thr Ile Ser Val Asp Arg Ser Lys Asn Gln Phe  
65 70 75 80

Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr  
85 90 95

Cys Ala Arg

<210> 49  
<211> 99  
<212> PRT  
<213> Homo sapiens

<400> 49  
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln  
1 5 10 15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile Ser Ser Gly  
20 25 30

Asp Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu  
35 40 45

Trp Ile Gly Tyr Ile Tyr Tyr Ser Gly Ser Thr Tyr Tyr Asn Pro Ser  
50 55 60

Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe  
65 70 75 80

Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr  
85 90 95

Cys Ala Arg

<210> 50  
<211> 99  
<212> PRT  
<213> Homo sapiens

<400> 50  
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln  
1 5 10 15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile Ser Ser Gly  
20 25 30

Gly Tyr Tyr Trp Ser Trp Ile Arg Gln His Pro Gly Lys Gly Leu Glu  
35 40 45

Trp Ile Gly Tyr Ile Tyr Tyr Ser Gly Ser Thr Tyr Tyr Asn Pro Ser  
50 55 60

Leu Lys Ser Leu Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe  
65 70 75 80

Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr

85

90

95

Cys Ala Arg

<210> 51  
 <211> 99  
 <212> PRT  
 <213> Homo sapiens

<400> 51  
 Gln Leu Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
   1                  5                  10                  15  
 Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile Ser Ser Ser  
                   20                  25                  30  
 Ser Tyr Tyr Trp Gly Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu  
                   35                  40                  45  
 Trp Ile Gly Ser Ile Tyr Tyr Ser Gly Ser Thr Tyr Tyr Asn Pro Ser  
           50                  55                  60  
 Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe  
   65                  70                  75                  80  
 Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr  
                   85                  90                  95

Cys Ala Arg

<210> 52  
 <211> 99  
 <212> PRT  
 <213> Homo sapiens

<400> 52  
 Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
   1                  5                  10                  15  
 Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Val Ser Ser Gly  
                   20                  25                  30  
 Ser Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu  
                   35                  40                  45  
 Trp Ile Gly Tyr Ile Tyr Tyr Ser Gly Ser Thr Asn Tyr Asn Pro Ser  
           50                  55                  60  
 Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe  
   65                  70                  75                  80  
 Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr  
                   85                  90                  95

Cys Ala Arg

<210> 53  
 <211> 119  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 protein construct

<400> 53  
 Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
   1                  5                  10                  15  
 Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
                   20                  25                  30  
 Gly Met Gly Val Gly Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
                   35                  40                  45  
 Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr Asn Pro Ser  
   50                  55                  60  
 Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val  
   65                  70                  75                  80  
 Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
                   85                  90                  95  
 Cys Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Gln Gly  
                   100                  105                  110  
 Thr Leu Val Thr Val Ser Ser  
                   115

<210> 54  
 <211> 119  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 protein construct

<400> 54  
 Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
   1                  5                  10                  15  
 Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
                   20                  25                  30  
 Gly Met Gly Val Gly Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
                   35                  40                  45  
 Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr Gln Pro Ser  
   50                  55                  60  
 Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val  
   65                  70                  75                  80



Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
                                     85                                    90                                    95

Cys Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Gln Gly  
                                     100                                    105                                    110

Thr Leu Val Thr Val Ser Ser  
                                     115

<210> 55

<211> 330

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
                                     protein construct

<400> 55

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys  
   1                                    5                                    10                                    15

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr  
                                     20                                    25                                    30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser  
                                     35                                    40                                    45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser  
   50                                    55                                    60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr  
   65                                    70                                    75                                    80

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys  
                                     85                                    90                                    95

Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys  
                                     100                                    105                                    110

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro  
                                     115                                    120                                    125

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys  
   130                                    135                                    140

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp  
   145                                    150                                    155                                    160

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu  
                                     165                                    170                                    175

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu  
                                     180                                    185                                    190

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn  
                                     195                                    200                                    205

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly

210		215		220
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu				
225		230	235	240
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr				
	245		250	255
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn				
	260		265	270
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe				
	275		280	285
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn				
	290		295	300
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr				
305		310	315	320
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys				
	325		330	

&lt;210&gt; 56

&lt;211&gt; 330

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
protein construct

&lt;400&gt; 56

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
1 5 10 15

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
50 55 60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
65 70 75 80

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
85 90 95

Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
100 105 110

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
115 120 125

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
130 135 140

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp  
 145 150 155 160  
 Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu  
 165 170 175  
 Glu Gln Tyr Ala Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu  
 180 185 190  
 His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn  
 195 200 205  
 Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly  
 210 215 220  
 Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu  
 225 230 235 240  
 Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr  
 245 250 255  
 Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn  
 260 265 270  
 Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe  
 275 280 285  
 Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn  
 290 295 300  
 Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr  
 305 310 315 320  
 Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys  
 325 330

&lt;210&gt; 57

&lt;211&gt; 107

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
 protein construct

&lt;400&gt; 57

Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu  
 1 5 10 15

Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe  
 20 25 30

Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln  
 35 40 45

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser  
 50 55 60

Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu



<210> 59  
 <211> 20  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 amino acid leader sequence

<400> 59  
 Met Glu Thr Gln Ser Gln Val Phe Val Tyr Met Leu Leu Trp Leu Ser  
     1                    5                    10                    15  
 Gly Val Asp Gly  
                     20

<210> 60  
 <211> 449  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 protein construct

<400> 60  
 Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
     1                    5                    10                    15  
 Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
                     20                    25                    30  
 Gly Met Gly Val Gly Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
                     35                    40                    45  
 Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr Asn Pro Ser  
     50                    55                    60  
 Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val  
     65                    70                    75                    80  
 Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
                     85                    90                    95  
 Cys Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Gln Gly  
                     100                    105                    110  
 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
     115                    120                    125  
 Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
     130                    135                    140  
 Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp  
     145                    150                    155                    160  
 Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu  
                     165                    170                    175



Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser  
 180 185 190  
 Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro  
 195 200 205  
 Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys  
 210 215 220  
 Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro  
 225 230 235 240  
 Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser  
 245 250 255  
 Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp  
 260 265 270  
 Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn  
 275 280 285  
 Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val  
 290 295 300  
 Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu  
 305 310 315 320  
 Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys  
 325 330 335  
 Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr  
 340 345 350  
 Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
 355 360 365  
 Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
 370 375 380  
 Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
 385 390 395 400  
 Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
 405 410 415  
 Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
 420 425 430  
 Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
 435 440 445

Lys

<210> 61  
 <211> 449  
 <212> PRT  
 <213> Artificial Sequence

<223> Description of Artificial Sequence: Synthetic protein construct

Gln 1	Val	Thr	Leu	Arg 5	Glu	Ser	Gly	Pro	Ala 10	Leu	Val	Lys	Pro	Thr 15	Gln
Thr	Leu	Thr	Leu 20	Thr	Cys	Thr	Phe	Ser 25	Gly	Phe	Ser	Leu	Ser 30	Thr	Ser
Gly	Met	Gly 35	Val	Gly	Trp	Ile	Arg 40	Gln	Pro	Pro	Gly	Lys 45	Ala	Leu	Glu
Trp	Leu 50	Ala	His	Ile	Trp	Trp 55	Asp	Asp	Asp	Lys	Tyr 60	Tyr	Asn	Pro	Ser
Leu 65	Lys	Ser	Arg	Leu	Thr 70	Ile	Ser	Lys	Asp	Thr 75	Ser	Lys	Asn	Gln	Val 80
Val	Leu	Thr	Met	Thr 85	Asn	Met	Asp	Pro	Val 90	Asp	Thr	Ala	Thr	Tyr 95	Tyr
Cys	Ala	Arg	Thr 100	Arg	Arg	Tyr	Phe	Pro 105	Phe	Ala	Tyr	Trp	Gly 110	Gln	Gly
Thr	Leu	Val 115	Thr	Val	Ser	Ser	Ala 120	Ser	Thr	Lys	Gly	Pro 125	Ser	Val	Phe
Pro	Leu 130	Ala	Pro	Ser	Ser	Lys 135	Ser	Thr	Ser	Gly	Gly 140	Thr	Ala	Ala	Leu
Gly 145	Cys	Leu	Val	Lys	Asp 150	Tyr	Phe	Pro	Glu 155	Pro	Val	Thr	Val	Ser	Trp 160
Asn	Ser	Gly	Ala	Leu 165	Thr	Ser	Gly	Val	His 170	Thr	Phe	Pro	Ala	Val 175	Leu
Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser 185	Ser	Val	Val	Thr	Val 190	Pro	Ser
Ser	Ser	Leu 195	Gly	Thr	Gln	Thr	Tyr 200	Ile	Cys	Asn	Val	Asn 205	His	Lys	Pro
Ser	Asn 210	Thr	Lys	Val	Asp	Lys 215	Lys	Val	Glu	Pro	Lys 220	Ser	Cys	Asp	Lys
Thr 225	His	Thr	Cys	Pro	Pro 230	Cys	Pro	Ala	Pro	Glu 235	Leu	Leu	Gly	Gly	Pro 240
Ser	Val	Phe	Leu	Phe 245	Pro	Pro	Lys	Pro	Lys 250	Asp	Thr	Leu	Met	Ile 255	Ser
Arg	Thr	Pro	Glu 260	Val	Thr	Cys	Val	Val 265	Val	Asp	Val	Ser	His 270	Glu	Asp
Pro	Glu	Val 275	Lys	Phe	Asn	Trp	Tyr 280	Val	Asp	Gly	Val	Glu 285	Val	His	Asn
Ala	Lys 290	Thr	Lys	Pro	Arg	Glu 295	Glu	Gln	Tyr	Ala	Ser 300	Thr	Tyr	Arg	Val

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu  
 305 310 315 320

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys  
 325 330 335

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr  
 340 345 350

Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
 355 360 365

Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
 370 375 380

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
 385 390 395 400

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
 405 410 415

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
 420 425 430

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
 435 440 445

Lys

<210> 62  
 <211> 449  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 protein construct

<400> 62  
 Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
 1 5 10 15

Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
 20 25 30

Gly Met Gly Val Gly Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
 35 40 45

Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr Gln Pro Ser  
 50 55 60

Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val  
 65 70 75 80

Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
 85 90 95

Cys Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Gln Gly

100						105						110					
Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe		
		115					120						125				
Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu		
		130					135						140				
Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp		
					150						155				160		
Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu		
				165					170					175			
Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser		
			180					185					190				
Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro		
		195					200						205				
Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys		
		210					215				220						
Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro		
					230					235					240		
Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser		
				245					250					255			
Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp		
			260					265					270				
Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn		
		275					280						285				
Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val		
		290					295				300						
Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu		
					310					315					320		
Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys		
				325					330					335			
Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr		
			340					345					350				
Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr		
			355				360						365				
Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu		
					375						380						
Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu		
					390					395					400		
Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys		
				405					410					415			
Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu		
			420					425					430				

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
 435 440 445

Lys

<210> 63

<211> 449

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
 protein construct

<400> 63

Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
 1 5 10 15

Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
 20 25 30

Gly Met Gly Val Gly Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
 35 40 45

Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr Gln Pro Ser  
 50 55 60

Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val  
 65 70 75 80

Val Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr  
 85 90 95

Cys Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Gln Gly  
 100 105 110

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
 130 135 140

Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp  
 145 150 155 160

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu  
 165 170 175

Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser  
 180 185 190

Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro  
 195 200 205

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys  
 210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro





<210> 65  
 <211> 50  
 <212> DNA

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 oligonucleotide

<400> 65  
 cacatttggt gggatgatga taagtactat caaccatccc tgaagagcca 50

<210> 66  
 <211> 138  
 <212> PRT  
 <213> Mus musculus

<400> 66  
 Met Asp Arg Leu Thr Phe Ser Phe Leu Leu Leu Ile Val Pro Ala Tyr  
     1                    5                    10                    15  
 Val Leu Ser Gln Val Thr Leu Lys Glu Ser Gly Pro Gly Ile Leu Lys  
                     20                    25                    30  
 Pro Ser Gln Thr Leu Ser Leu Thr Cys Ser Phe Ser Gly Phe Ser Leu  
                     35                    40                    45  
 Ser Thr Ser Gly Met Gly Val Gly Trp Ile Arg Gln Pro Ser Gly Lys  
     50                    55                    60  
 Gly Leu Glu Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Tyr  
     65                    70                    75                    80  
 Gln Pro Ser Leu Lys Ser Gln Leu Thr Ile Ser Lys Asp Thr Ser Arg  
                     85                    90                    95  
 Asn Gln Val Phe Leu Lys Ile Thr Ser Val Asp Thr Ala Asp Ala Ala  
                     100                    105                    110  
 Thr Tyr Tyr Cys Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp  
     115                    120                    125  
 Gly Gln Gly Thr Leu Val Thr Val Ser Ser  
     130                    135

<210> 67  
 <211> 118  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
 protein construct

<400> 67  
 Gln Val Thr Leu Lys Glu Ser Gly Pro Gly Ile Leu Gln Pro Ser Gln  
     1                    5                    10                    15

Thr Leu Ser Leu Thr Cys Ser Phe Ser Gly Phe Ser Leu Ser Thr Ser  
                     20                    25                    30  
 Gly Met Gly Val Gly Trp Ile Arg Gln Pro Ser Gly Lys Gly Leu Glu  
                     35                    40                    45  
 Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Asn Pro Ser Leu  
                     50                    55                    60  
 Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Ser Asn Gln Val Phe  
                     65                    70                    75                    80  
 Leu Lys Ile Thr Ser Val Asp Thr Arg Asp Thr Ala Thr Tyr Tyr Cys  
                     85                    90                    95  
 Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Glu Gly Thr  
                     100                    105                    110  
 Ser Val Thr Val Thr Ser  
                     115

<210> 68  
 <211> 118  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Synthetic  
           protein construct

<400> 68  
 Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
   1                    5                    10                    15  
 Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
                     20                    25                    30  
 Gly Met Gly Val Gly Trp Ile Arg Gln Pro Pro Gly Lys Ala Leu Glu  
                     35                    40                    45  
 Trp Leu Ala His Ile Trp Trp Asp Asp Asp Lys Tyr Asn Pro Ser Leu  
                     50                    55                    60  
 Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val Val  
                     65                    70                    75                    80  
 Leu Thr Met Thr Asn Met Asp Pro Val Asp Thr Ala Thr Tyr Tyr Cys  
                     85                    90                    95  
 Ala Arg Thr Arg Arg Tyr Phe Pro Phe Ala Tyr Trp Gly Gln Gly Thr  
                     100                    105                    110  
 Leu Val Thr Val Ser Ser  
                     115  
 TLN-029PC